ULTRASTRUCTURE AND FUNCTION OF RESPIRATORY CILIA IN CRITICAL ILLNESS

By

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A thesis submitted to the University of Plymouth in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

(August 2004)

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Abstract

Approximately one in eleven hospital patients suffer a nosocomial infection. Intensive care patients are at particular risk and nosocomial pneumonia especially is a problem in the intensive care unit (ICU). The mucociliary transport system is the major defence mechanism against such infection and whilst its efficiency has been shown to be decreased in ICU patients there are few reports in the literature describing the ultrastructure of the respiratory epithelium in this patient group. The principal aims of this study included an investigation of the ultrastructure of the respiratory ciliated epithelium in ICU patients, together with an assessment of the impact of the known risk factors anaesthesia and cigarette smoking on the fine structure and function of the mucociliary transport system.

Duration of anaesthesia is a risk factor for infection in the ICU and several anaesthetic agents have been shown to impair mucus transport rate (MTR) and ciliary beat frequency (CBF). Rat tissue was used to investigate the effect of the intravenous anaesthetics midazolam and propofol on ciliary survival, whilst the effect of the gas halothane was observed on human cell cultures using high speed digital video recording. There was no deleterious effect of the two intravenous anaesthetic agents on ciliary survival, assessed using a semi-automated image analysis system. However, the damaging effect of halothane was confirmed and associated with novel findings of decreased amplitude and synchrony, as well as cilia beat frequency (decrease of approximately 30%). In contrast to previous reports the study of a large sample of asymptomatic smokers and non-smokers revealed no difference in ciliary abnormalities between the two groups (the proportion of abnormal cilia in each was approximately 3%).

The evaluation of ciliary structural abnormalities by transmission electron microscopy confirmed there was wide variation in their occurrence among critically ill patients and that it was imperative, contrary to previous reports, to record a large number of cilia from a range of fields owing to the localised effect of some abnormalities. The present investigation was also the first to make observations on ciliary abnormalities beyond three days admission to the ICU thus providing new information on the ultrastructure of respiratory cilia in long stay ICU patients, potentially leading to improved treatment protocols for this patient group.

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'....the general existence of the ciliary motion in the Animal Kingdom is already sufficiently established,...whoever has opportunities and inclination to cultivate this field of inquiry will find his labour rewarded by much curious and interesting discovery.'

Sharpey, 1835

From Sleigh (1962)

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Acknowledgements

Chapter 5. Ross Kay, another PhD student, helped me by processing 20 of the samples.

Chapter 6. I collaborated with Ross Kay, to investigate specimens from critically ill patients. My contribution was to process and analyse the first 18 ICU samples for cilial abnormalities by transmission electron microscopy, whilst Ross Kay processed and analysed these samples for cilial abundance with scanning electron microscopy. My own data (Chapter 6) are mentioned in Ross Kay's PhD dissertation as a baseline comparative against his own subsequently collected specimens which are separately reported.

I would also like to thank the following people for their invaluable support and assistance:

Dr Roy Moate, Professor Rob Sneyd, and Dr Jeremy Langton for their continuous guidance, support and perseverance. Dr Susan Loxdale and Dr Pete Macnaughton for their time during collection of human samples. Dr Markus Rautiainen and Dr Elina Toskala for their welcome and support during my stay in Finland. Dr Chris Ricketts and David Wright for invaluable statistics advice (before, during and after!).

To all the staff at the University of Plymouth who have provided an excellent source of practical advice.

Presentations, publications, and conferences attended.

Oral presentations

Industrial Histology Discussion Group. Daventry, October, 1998

J. Kirkby. Ultrastructure and function of respiratory cilia in the critically ill. Ultrastructural Pathology (proceedings) *(awarded annual Ray Dank's Award for best short talk or new speaker.)*

Anaesthetic Research Society. London, November, 1998

Kirkby. J, Russell. P, Sneyd. J R, Moate. R M, Langton. J A. Electron microscopy of respiratory ciliated epithelium. British Journal of Anaesthesia, 82, (3) 464-465

Anaesthetic Research Society. Plymouth, July, 1999

Kirkby. J, Sneyd. J R, Langton. J A, Moate. R M. 1999. Effect of midazolam on cilia survival. British Journal of Anaesthesia, 83, 527P

The Biochemical Annual Conference. Birmingham, March, 2000

Kirkby. J, Kay. R, Sneyd. J R, Langton. J A, Moate. R M. The mucociliary transport system.

(awarded 2nd prize for best oral presentation)

Anaesthetic Research Society. London, November, 2000

Kay. R, Kirkby. J, Watson. G, Sneyd. J R, Langton. J A, Moate. RM, Macnaughton. P

Ciliary abundance and abnormalities in critical illness. (in press, BJA)

Poster presentations

The Biochemical Annual Conference. Birmingham, March, 2000

Kay. R, Kirkby. J, Moate. RM, Sneyd. J R, Langton. J A. Ultrastructure and function of respiratory ciliated epithelium in critical illness.

Anaesthetic Research Society. London, November, 2000

Kirkby. J, Kay. R, Sneyd. J R, Langton. J A, Moate. R M, Loxdale. S. Ciliary abnormalities in non-symptomatic smokers and non-smokers. (in press, BJA)

(awarded ARS prize for best presentation)

Other conferences attended

- Micro 2000. London, April, 2000
- Derriford Infection Control Meeting. May, 2000
- The Pathological Society of Great Britain and Northern Ireland. Nottingham, September, 2000
- Local Defence Mechanisms in ENT. Roeselare, Belgium. October, 2000

Signed...J...KIRKBY.....

Dated...NOVEMBER...04...

Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was funded by a Faculty of Science studentship and Anaesthesia Research Plymouth.

A programme of advanced research was undertaken, which included laboratory based research at the University of Plymouth and clinical sample collection at the Freedom Day Case Unit and the Intensive Care Unit at Derriford Hospital, Plymouth.

Relevant scientific seminars and conferences were regularly attended at which several abstracts were presented.

Signed.....

Dated.....

Abbreviations

ACh	Acetylcholine
ATP	Adenosine triphosphate
APACHE	Acute physiology and chronic health evaluation
BTV	Bronchial mucus transport velocity
cAMP	Cyclic adenosine monophosphate
CBA	Cilia beat amplitude
CBF	Cilia beat frequency
CC	Central complex [of cilia]
cGMP	Cyclic guanine monophosphate
Ci	Cilia
CO_2	Carbon dioxide
CSP	Chronic sputum production
CT	Cholera toxin
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified eagle's medium
EPIC	European Prevalence of Infection in Intensive Care (study)
f	Female
g	Grams
HAI	Hospital acquired infection
HRE	Human respiratory epithelial
Hz	Hertz
i	Inner dynein arm
ICU	Intensive care unit

IU	International Units
kV	kilovolts
LM	Light microscopy
M	Molar
m	Male
M199	Medium 199
MAb	Monoclonal antibody
MAC	Minimum alveolar concentration
mg	milligrams
MTR	Mucus transport rate
Mu	Mucus
n	Nexin
NCS	Newborn calf serum
nm	nanometer
NNIS	National Nosocomial Infections Surveillance System (USA)
NO	Nitric Oxide
NOS	Nitric Oxide synthase
Nu	Nucleus
o	Outer dynein arm
O_2	Oxygen
p	projections associated with central pair of microtubules
PCD	Primary ciliary dyskinesia
PKA	Protein kinase
r	Radial spokes
RA	Retinoic acid

RTE	Rat tracheal epithelial
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
µm	micrometer
Q-570	Quantimet-570 (image analyser)
VAP	Ventilator associated pneumonia

Glossary

Autolysis

Spontaneous lysis (rupture) of cells or organelles produced by the release of internal hydrolytic enzymes. Normally associated with the release of lysosomal enzymes.

Axoneme

The central microtubule complex of eukaryotic cilia and flagella with the characteristic 9 + 2 arrangement of tubules when seen in cross-section.

Basal body

Structure found at the base of eukaryotic cilia and flagella consisting of a continuation of the nine outer sets of axonemal microtubules but with the addition of a C-tubule to form a triplet (like the centriole). Anchored to the cytoplasm by rootlets

Bronchiectasis

Dilation of the bronchi or bronchioles. May be congenital or result from infection (lung disease) or obstruction (cancer).

Ciliostasis

Cessation of ciliary beating

Dynein

Large multimeric protein (600 – 800kD) with ATPase activity, constitutes the side arms of

the outer microtubule doublets in the ciliary axoneme and responsible for the sliding movement, which results in ciliary beating.

Goblet cell

Goblet-shaped, mucus-secreting epithelial cell, found in the respiratory tract.

Glycoprotein

Proteins with covalently attached carbohydrate residues. Includes most secreted proteins.

Hyperplasia

Increased number of cells in a tissue or organ, which can be reflected in an increase in organ size. Can occur during wound healing or after mechanical stress.

Metaplasia

Abnormal change in the phenotype of a tissue. For example, the change of columnar epithelium in the respiratory tract to squamous epithelium as a result of chronic damage.

Microtubule

Cytoplasmic tubule, 25nm outside diameter with a 5nm thick wall. Made of tubulin heterodimers.

Microvilli

Projections from the apical surface of an epithelial cell that are supported by a central core of microfilaments associated with bundling proteins such as villin and fimbrin.

Mucous

Covered with or secreting mucus.

Mucus

A viscoelastic secretion, rich in mucins that is secreted by mucous membranes and that serves to moisten and protect such membranes.

Nosocomial infection

An infection that is not present or incubating when the patient is admitted to hospital or other health care facility. Usually manifests between 48 - 72 hours after admission. Derived from the Greek words nosos [disease] and komein [to care for] and later the Latin word for hospital, nosocomium.

Primary Ciliary Dyskinesia (PCD)

Congenital disorder of ciliary movement. Symptoms include bronchiectasis and chronic sinusitis.

Pseudostratified epithelium

Epithelium consisting of a single layer of cells of different heights, not all reaching the open surface. The nuclei are therefore seen at different levels and give the false or pseudo appearance of the epithelium being stratified. In fact all of the cells reach the basement membrane.

Situs invertus

Condition in which the normal asymmetry of the body (in respect of the circulatory system

and intestinal coiling) is reversed. Occurs in approximately 50% of patients with PCD.

Tubulin

Cytoplasmic protein (55kD) found mainly in two forms, alpha and beta. Heterodimers of α and β tubulin form subunits of microtubules.

CHAPTER ONE

General Introduction

1 The mucociliary transport system

The respiratory tract (Figure 1.1) is lined by pseudostratified columnar ciliated epithelium (Figure 1.2) which forms part of the mucociliary transport system, a vital defence mechanism against respiratory infections. The major components of the mucociliary transport system are the cilia and the overlying mucus. The ratio of ciliated cells: goblet cells is about 5:1 with numbers of both decreasing from the trachea to the peripheral airways (Wanner et al., 1996). Goblet cells, found at the surface epithelium are usually mucous cells, which hold granules containing tightly packed high molecular weight glycoproteins (mucins) with sialic acid and sulphate groups, the principal constituents of mucus. Secretory cells of the submucosal glands may be mucous or serous cells. The intracellular granules of serous cells hold smaller neutral glycoproteins and may also contain lipids, lysozyme, lactoferrin, and mucus proteinase inhibitor. Inhaled particulate material and microorganisms are trapped in the mucus which is transported by the cilia away from the tracheobronchial tree towards the oesophagus where it is swallowed, preventing any adhesion and migration through the epithelial cells.

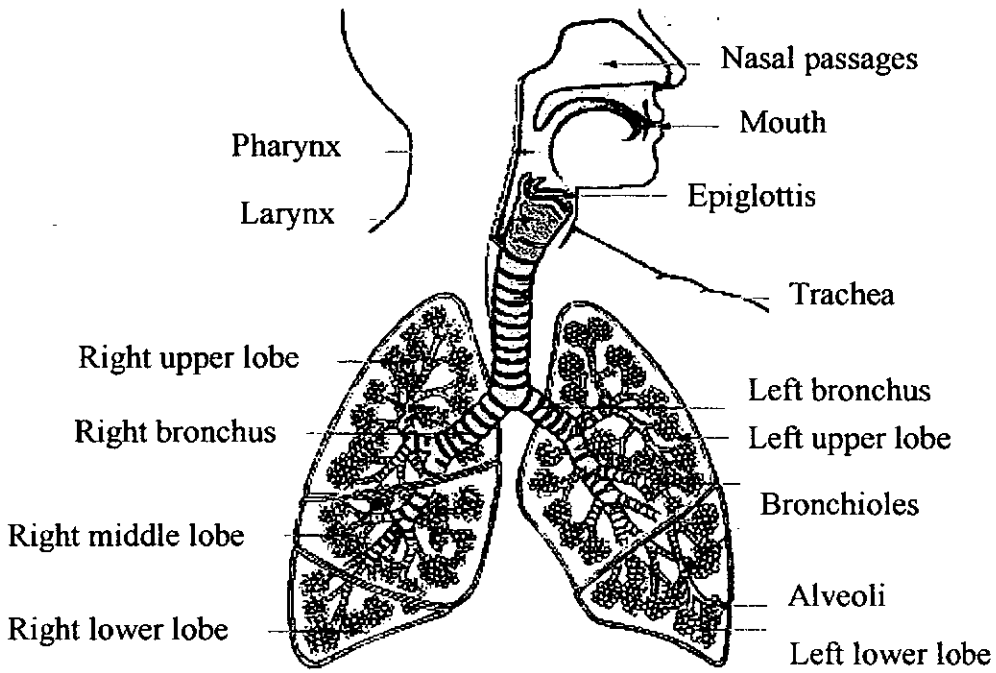


Figure 1.1 Outline of the respiratory tract

Adapted from diagrams in: http://www.lungsusa.org and http://www.mtsinai.org/pulmonary

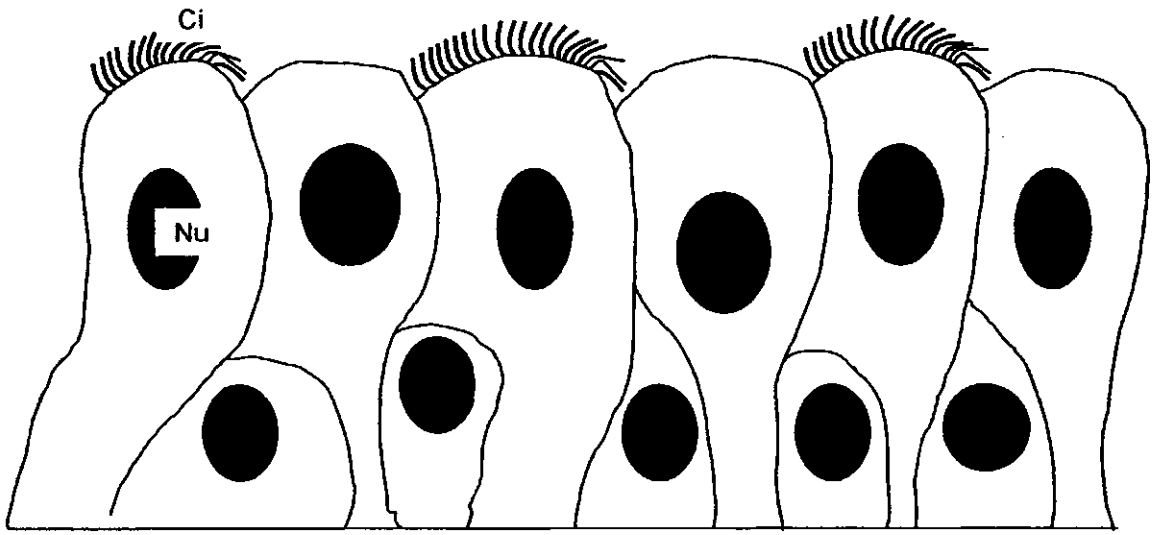


Figure 1.2 Pseudostratified epithelium of the respiratory tract

In pseudostratified epithelia there appear to be many layers of cells due to the different nuclear layers. In fact, all cells rest on the basement membrane though not all reach the surface.

Ci Cilia
Nu Nucleus

Non-ciliated cells may contain microvilli that are about 1μm in length and between 0.1 – 0.3 μm in diameter. It is thought that these may represent growing or degenerating cilia (Wanner, et al., 1996). The continuous process of regeneration maintains the epithelium but cell turnover is slow: <1% of cells proliferate within 24 hours, though this process speeds up after injury, after which 17% of cells are believed to be undergoing proliferation (Ayers and Jeffery 1988). Basal cells differentiate into mucous cells, which subsequently develop into ciliated cells. Basal cells have been shown to have a turnover rate of between 3 and 38 weeks but little is known about the life span of other epithelial cells (Plopper et al., 1992). After mild mechanical injury, epithelial regeneration begins within 12 hours and is complete within 2 weeks. After more severe damage it takes between 2-4 days for the regeneration process to begin and up to 29 days to be completed (Hilding 1964 et al., 1972; Shimizu et al., 1994).

1.6 Cilia

Cilia are hair-like projections with an average length of 6 μm and a diameter of 0.1 μm to 0.2 μm. Found throughout the respiratory tract, ciliated cells have an average of around 200 cilia per cell. The oldest known cell organelles, cilia, were first described by the Dutch microscopist Antony van Leewenhoek in September 1675 (Dobell 1958), whilst O. F. Muller was the first to use the name cilia, meaning eyelash in 1786 (Muller 1786). By 1835 cilia had been observed in most of the main animal groups, including mammals in 1834 (Sleigh 1962). Grant (1835) suggested that cilia might move by the flowing of water into and out of them (Grant 1835) while others of the time including Ehrenberg (1832), Sleigh 1962; Satir 1995, and Purkinji and Valentin (1835). Purkinji and Valentin thought that cilia were moved by small muscles at their base. Sharpey (1835) found no evidence of this and suggested that cilia actually 'contain a muscular substance throughout a greater or less

part of their length, by which they can be bent or extended' (Sharpey 1835-36). Sharpey had seen that cilia bend in the main part of their length as well as at the base and was first to describe the metachronal waves, which he likened to those produced by wind in a corn field.

Koltzoff (1903) thought that cilia had an organisation of internal filaments and this was later confirmed (Dellinger 1909). These early observations of cilia were limited by the inadequate resolution of the light microscope and it wasn't until the advent of electron microscopy in the 1950s that detailed ultrastructure could finally be elucidated. The cilium has a structure consisting of a '9+2' arrangement of microtubule doublets known as the axoneme (Figure 1.3). This distinct arrangement was first demonstrated by Manton (1952) in the cilia of plant cells. The outer nine doublets are made up of a complete A subfibre, which consists of thirteen tubulin dimer protofilaments, and an incomplete B subfibre consisting of ten to eleven tubulin dimer protofilaments. Tetkin is found in the region where the two subfibres come together and provides structural stability of microtubules. The nine outer doublets are connected to each other by means of paired nexin links and to projections from the two inner microtubules by radial spokes. The microtubules are made up principally of α- and β- tubulin. Afzelius (1959) first described the dynein arms projecting from the outer microtubule doublets. The A subfibres have an outer and an inner dynein arm projecting in a clockwise direction towards the B subfibre as described by Gibbons and Grimstone (1960). Fawcett and Porter (1954) showed that the entire axoneme is enclosed by a membrane that is continuous with the plasma membrane of the cell.

At the base of the cilium, in the basal body, the doublets are replaced by triplets which gradually taper off. The central microtubules terminate at the level of the cell and do not

descend into the basal body. In the tip of the cilium the doublets become single and decrease in number to a single A fibre (Figure 1.4). From a dense cap at the tip of the cilium project 3-7 short claws 25-35nm long which are thought to anchor the overlying mucus (Wanner et al., 1996).

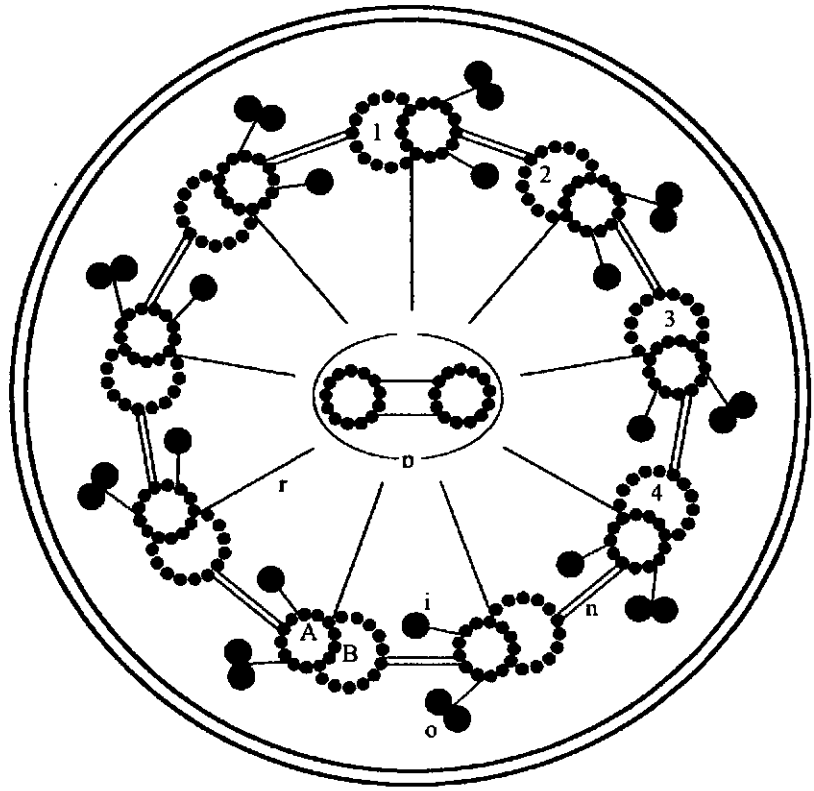
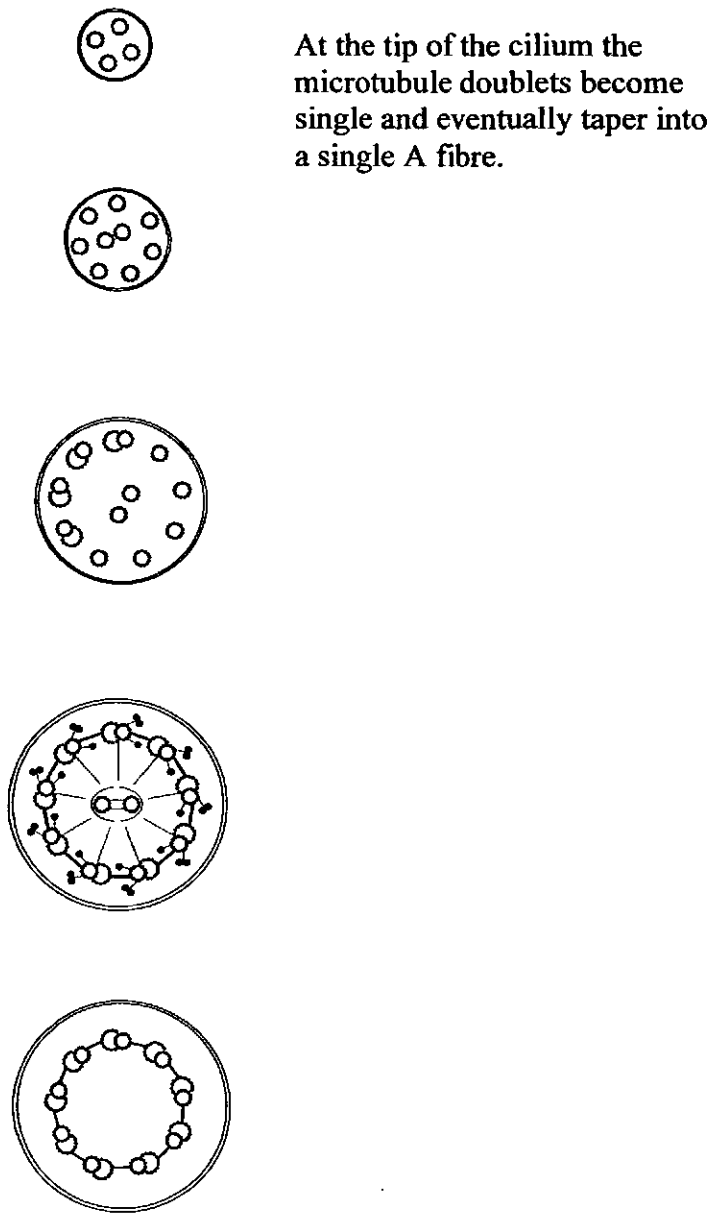


Figure 1.3 Schematic diagram of a normal cilium in transverse cross-section showing conventional numbering of outer doublets.

n	**nexin**
i	**inner dynein arm**
o	**outer dynein arm**
r	**radial spokes**
p	**projections associated with central microtubules**

The inner dynein arms of the cilium are thought to regulate beat formation, and the outer dynein arms to regulate CBF. Sliding of the outer doublets is powered by the ATPase outer dynein arms. This results in bending of the cilium because the doublets are prevented from sliding apart by the presence of the nexin links and radial spokes. The radial spokes connect the outer doublets with projections associated with the central pair microtubules, maintaining the axonemal structure.

Tip



At the tip of the cilium the microtubule doublets become single and eventually taper into a single A fibre.

The microtubules descend into the basal body, becoming triplets in the cell cortex.

Base

Figure 1.4 Schematic diagram showing structure of a normal cilium as it changes from base to tip.

Cilia beat in a coordinated fashion at a frequency of about 12 Hz and the beating cycle is illustrated in Figure 1.5. Each dynein head contains a heavy chain ATPase. These dynein heads are attached to several dynein light chains and an intermediate chain which anchor the arm to the A sub fibre. Movement of the cilium is brought about by the formation of molecular bridges between adjacent A and B subfibres and the hydrolysis of ATP. The hydrolysis of ATP enables the doublet microtubules to undergo a conformational change causing them to slide relative to each other. The nexin links between the microtubule doublets prevent them from simply sliding apart, thus causing the cilium to bend. It is thought that the outer dynein arms regulate CBF while the inner dynein arms regulate bend formation and beat form (Wanner et al., 1996). Microtubule sliding has been demonstrated (Satir 1965) but the exact mechanism by which the cilium bends in two directions (during effective and recovery strokes) is not fully understood. The switch-point hypothesis put forward by Satir appears to be the best supported theory (Wanner et al., 1996; Satir and Matsuoka, 1989) and suggests that the dynein arms of one side of the axoneme are active only during the effector stroke (arms of microtublule doublets 1 – 4) while those of the other side (arms of microtubules 6 – 9) are active only during the recovery stroke.

The beating cycle consists of an effective stroke, a resting state, and a recovery stroke. During the effective stroke a velocity of 1mm/ s may be reached at the tip. It was thought until recently that during the recovery stroke, when the cilium bends back almost 180°, it swung out sideways close to the surface of the cell. Using high speed digital video however, the actual angle of deviation from the plane of the effective stroke during the recovery stroke was found to be minimal. (Chilvers, 2000).

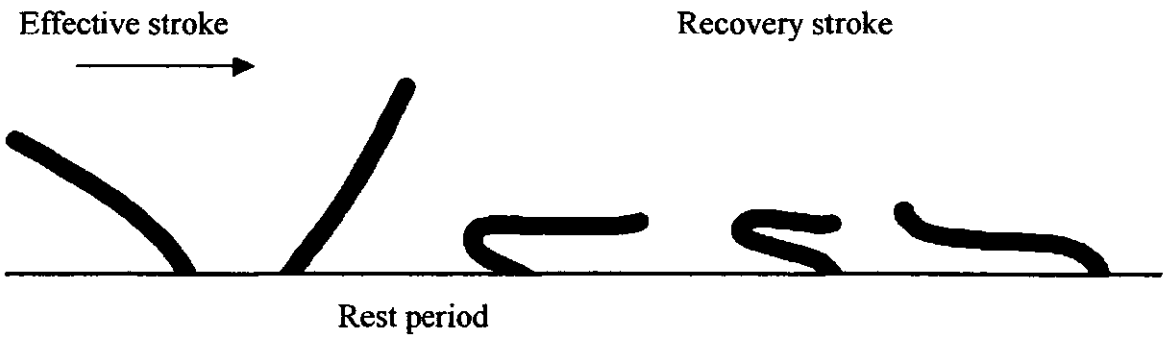


Figure 1.5 Motion of ciliary beat as seen from the side

During the effective stroke the cilium reaches a speed of 1 mm/ second and, fully extended, engages the overlying mucus. After the effective stroke, the cilium rests before resuming the recovery stroke during which it swings back about 180°.

1.7 Mucus

Respiratory mucus traps inhaled particulate matter and micro-organisms and is then transported away by the cilia. A hydrophilic, non-Newtonian fluid (i.e. viscosity decreases as applied force increases), it contains various glycoproteins (including mucins) and immunologic substances. It is composed of two layers, the periciliary layer (sol phase) and the mucus layer (gel phase). The periciliary layer lies close to the cilia, about as deep as the cilia are long. The low viscosity of the periciliary layer enables the cilia to beat freely and thus its depth is critical to optimal mucus transport. If the level of the periciliary layer is too high, the tips of the cilia would not come into contact with the overlying mucus layer whereas if this layer is too low, the cilia would become impeded by the mucus. Carbohydrate-rich glycoproteins or mucins are a key component of mucus. Mucins consist of oligosaccharide units attached to a long polypeptide chain. The oligosaccharide side chains account for up to 80% of the mucin weight. Mucins link to each other at each end via disulphide bonds to form long molecules. Stored in a condensed form, mucins are hydrated upon exocytosis when the secretory granule fuses with the cell membrane. This fusion results in the formation of a secretory pore and exchange of calcium ions inside the granule with extracellular sodium ions. This exchange triggers rapid mucin hydration (Houtmeyers et al., 1999). Mucin hydration is important in the regulation of the rheological properties of mucus since these are mainly determined by mucin concentration. The hydration of mucin is therefore critical, since the ability of mucus to trap foreign particles and of the cilia to transport the mucus depends upon its rheological properties.

A healthy adult expels between 10 – 100 ml of mucus, produced in airway goblet and submucosal gland cells, per day. This is propelled at a rate of 0.2mm sec^{-1} towards the

oesophagus where it is swallowed (Wanner et al., 1996).

1.8 Mucus transport rate (MTR)

The rate of mucus transport can be measured in various ways and results obtained are dependent on the invasiveness of the technique and the area of the respiratory tract studied. For example, a dye can be placed on the nasal mucosa and the time taken to reach the nasopharynx recorded. The time from placement of sodium saccharide particles to the subject reporting a sweet taste can also be measured. Teflon discs can be deposited and viewed directly through a bronchoscope or radio-labelled particles can be inhaled and viewed using a gamma camera as a less invasive means of measurement (Wanner et al., 1996). MTR is highest in the trachea and decreases with increasing airway generation. Normal MTR in the human trachea is approximately 4 to 5 mm/ min as measured with a minimally invasive technique and up to 20 mm/ min as measured with an invasive technique. Efficient mucus transport is dependent upon the interaction between the beating cilia and the overlying mucus. Cilia beat frequency is the main factor determining MTR with small changes in beat frequency associated with large changes in MTR as illustrated in a study by Duchateau et al (1985).

1.8.1 Measurement of cilia beat frequency

1.8.1.1 In vitro

Early methods of measuring the activity of cilia were based upon observing the rate of work done by the cilia. Speed of movement of particles such as carbon over a ciliated epithelium, or rate of rotation of a small cylinder held in contact with a ciliated epithelium, for example. Direct microscopic observation often involved subjectively describing ciliary motility as 'fast', 'slow', or 'stopped'. In the 1880's a stroboscope was used and high

speed cinematography came into use later. In 1962 Dalhamm and Rylander used a photosensitive cell to record the changes of light intensity reflected off the surface of ciliated epithelium due to the moving cilia (Dalhamn 1962). This was used as an indirect measurement of beat frequency. Subsequently, this method was modified such that transmitted rather than reflected light is detected and, now known as the transmitted light technique is in common use (Yager et al. 1978). The transmitted light technique is objective, reproducible, and convenient to use but have the disadvantage that it is sensitive to unrelated vibrations and may only be able to measure CBF of groups of cells rather than of single cell (Rusznak et al 1994). More recently, technological advances lead to the use of high speed digital video; it is now possible to record images of beating cilia at a rate of 400 frames per second. High speed digital video has been compared with the photomultiplier and photoelectric techniques with no significant difference in beat frequency measurements (Chilvers and O'Callaghan 2000). Although the transmitted light technique is reliable, high speed digital video allows beat pattern as well as frequency to be studied. Amplitude, co-ordination and orientation of the beat are also important determinants of MTR and can be studied in detail by viewing the recording at a much slower speed, or even frame by frame. This technique has been used to show the emergence of the cilial beat pattern in cell cultures (Rautiainen et al. 1992) and that the recovery stroke of the cilium does in fact take place in a plane close to that of the effective stroke (Chilvers and O'Callaghan 2000).

1.8.1.2 In vivo

Laser light scattering spectroscopy analyses changes in light intensity due to moving cilia and was first developed *in vitro* (Lee and Verdugo 1976) though it has since been used *in vivo* in pigs and dogs with results comparable to those *in vitro* (Svartengren et al. 1989;

Chandra et al. 1994). Verdugo and Golborne (1988) developed a fibre optic laser Doppler spectrometer that used a single fibre to transmit the laser light and collect the light scattered by beating cilia. This technique was also used in animals but the authors suggested that the fibre could be passed down a bronchoscope for use in humans. Huberman (1993) presented another *in vivo* method for use in humans, based on a fibre optic system whereby the light reflected off one fibre from the cilia is detected by another fibre, which could also be used with a bronchoscope. Results from fifteen adults appeared to be comparable to CBF *in vitro*. More recently, Paltieli et al (1997) constructed a device comprising a thin fibre optic probe with which to measure mucociliary wave frequency in the human nose, which they claimed caused no discomfort to the patient. These *in vivo* techniques have the advantage of measuring the CBF of cilia in their natural physiological environment and may be a useful diagnostic tool. However, information about beat pattern and form can only be obtained by high speed digital video recordings *in vitro*.

1.9 Control of cilia beat frequency

The precise mechanism by which cilia beat frequency in humans is controlled is unclear (Wanner et al 1996). This may be in part due to the difficulties in the measurement of the CBF of individual cells simultaneously with that of potential mediators. Both phosphorylation and calcium are known to regulate CBF as outlined below.

1.9.1 Phosphorylation

Increased levels of intracellular of cyclic adenosine monophosphate (cAMP) have been shown to increase CBF (DiBenedetto et al. 1991) whereas those of cyclic guanine monophosphate (cGMP) are associated with conflicting results. It is likely that the increase of CBF by increasing cAMP is via cAMP dependent protein kinase (PKA) activation since PKA has been shown to phosphorylate specific axonemal targets (Hamasaki et al. 1989). One such target (p29) has been identified as a regulatory light chain of the outer dynein arm in *Paramecium* and phosphorylation of p29 has been shown to increase the swimming speed of *Paramecium* (Hamasaki et al. 1991). Axonemal targets of PKA have also been found in other species and in mammals cAMP induces the phosphorylation of p26 (Salathe et al. 1993). In addition, protein kinase C has been found to phosphorylate a ciliary protein (p37) causing a decrease of CBF in mammals (Kobayashi et al. 1992).

1.9.2 Intracellular Ca^{2+}

CBF has long been known to be affected by intracellular free calcium concentration ($[Ca^{2+}]_i$). Lansley et al (1999) measured CBF and $[Ca^{2+}]_i$ simultaneously and found that when a mechanically induced $[Ca^{2+}]_i$ wave reached the ciliary base, CBF rapidly increased to a maximum of 26.7 Hz at 37 °C and could not be increased by further increasing the $[Ca^{2+}]_i$ by more than 250-300nM. Ca^{2+} may act via calmodulin by activation of a Ca^{2+}/

calmodulin dependent kinase or phoshatase, for example, ciliary calmodulin binding sites have been found in hamster respiratory epithelium (Gordon et al. 1982). Calmodulin may act directly or through an independent pathway. Some reports suggest a role for activation of a calmodulin-dependent nitric oxide synthase (NOS) though its role is unclear from the literature (Salathe and Bookman, 1995; Tamaoki et al, 1995; Wanner et al, 1996) and more research in the area is needed. However, in an attempt to elucidate the mode of cytoplasmic Ca^{2+} action, Salathe et al (1999) analysed the kinetics of coupling between $[Ca^{2+}]_i$ and CBF simultaneously from single ovine cells in response to Acetylcholine (ACh) and a calmodulin inhibitor. They found there was no significant delay between the increase in $[Ca^{2+}]_i$ and change in CBF. It was $\leq$ 1 beat cycle and although baseline CBF decreased with the addition of a calmodulin inhibitor, this coupling was not affected. The authors concluded that, in ovine cells, the timing of the Ca^{2+} action on CBF was suggestive of a direct mode of action rather than involvement of calmodulin-dependent phosphorylation or kinase/ phoshatase reactions. It is likely that there may be more than one independent mechanism and it is possible that these show species differences (Wanner et al. 1996).

1.9.3 Autonomic control

Airway secretion and mucociliary clearance are under autonomic control to a certain extent with the mucociliary apparatus being primarily innervated by the parasympathetic nervous system. For example, nerve fibers have been observed between ciliated cells in human bronchi viewed by electron microscopy (Laitinen 1985). Many autonomic agonists such as acetylcholine have been shown to have ciliostimulatory effects (Hybbinette and Mercke 1982; Gatto 1993). Several neurotransmitters, such as salbutamol, have also been shown to have secretory effects with increases in secretion rate and volume, and even an increase in the number of goblet cells (Jones and Reid 1979). Mucociliary activity is stimulated by

cholinergic and ß–adrenergic agonists such as acetylcholine, metacholine, and salbutamol (Gatto 1993) but overall autonomic control of ciliary activity and mucociliary clearance is still unclear.

1.5 Structure-function relationship in cilia

Structural integrity of the ciliary axoneme is critical for effective mucociliary transport to take place. Its importance becomes most apparent in conditions in which it is deficient.

1.6 Genetic abnormalities

Kartagener and Horlacher (1936) were first to describe a familial form of bronchiectasis with dextrocardia and nasal polyps. In 1976 Afzelius discovered a genetic disease caused by immotile cilia, now known as immotile cilia syndrome of which Kartagener's syndrome is a subgroup. Afzelius (1976) was also the first to discover that the cilia from these patients lacked dynein arms and that this defect was usually present in all cilia of the body. The main defect is usually lack of dynein arms but this is commonly accompanied by other acquired abnormalities, such as central pair or peripheral microtubular anomalies (Afzelius 1998). There are several other subgroups in which the symptoms and ultrastructural observations differ. For example, patients in whom the cilia have some movement have been described as having primary ciliary dyskinesia (PCD). This would include patients in whom cilia are ultrastructurally normal but are disorientated and those of others that lack the central pair of microtubules. Another defect is a total lack of cilia (acilia syndrome). Symptoms of all these may differ in severity and can include: chronic cough and expectoration of mucoid, mucopurulent sputum, bronchiectasis, chronic rhinitis, sinusitis, nasal polyposis, and recurrent maxillary rhinitis (Afzelius 1998).

1.7 Acquired abnormalities

Many different types of ultrastructural abnormality have been described, the heterogenous nature of which implies that these are acquired rather than of genetic origin (Afzelius 1976; Sturgess et al. 1980; Fox, Bull et al. 1983; Rossman et al. 1984; Afzelius et al. 1985; Carson et al. 1985; Afzelius 1998; Tamalet et al. 2001). It is acquired abnormalities, which are of particular interest in the research presented here. Cilial abnormalities have been described in association with several conditions including recurrent respiratory tract infections in children (Carson, Collier et al. 1985), also in smokers with bronchitis (Lungarella, Fonzi et al. 1983). The types, origin and functional significance are described below:

Figure 1.6 illustrates the ultrastructural anomalies commonly described in both healthy and diseased subjects.

1.7.1 Compound cilia

This abnormality is characterised by the presence of multiple axonemes within a common ciliary membrane. There are said to be two types according to the arrangement of the axonemes (Takasaka et al. 1980). Type 1 (adhesive) compound cilia, formed by the fusion of existing cilia, contain densely arranged axonemes whereas type 2 (bulging) compound cilia contain loosely and randomly embedded axonemes and are formed during ciliogenesis (Takasaka et al. 1980; Hagiwara et al. 2000). Compound cilia have been found in healthy and diseased subjects and can also be induced experimentally by chemicals such as SO_2 (Carson, Collier et al. 1987), and inflammatory mediators such as elastase (Fonzi et al. 1982). Compound cilia are also seen in conditions such as asthma, allergic rhinitis and chronic sinusitis, bronchial carcinoma and in smokers (Rossman et al. 1983). However, the

functional relevance of compound cilia is unclear. The incidence of compound cilia has not been found to differ between PCD and normal subjects, in contrast to peripheral and central defects. Torkkeli and colleagues (1997) measured mucociliary transport rate and examined the ultrastructure of cilia in patients with various respiratory symptoms. They found no correlation between the amount of compound cilia and MTR in their patients (Torkkeli et al. 1997). In contrast, McAuley and Anand (1998) described a patient with symptoms of chronic rhinitis in whom an increased number of compound cilia was associated with decreased mucociliary clearance (McAuley and Anand 1998). It should be noted though that no cause/ effect relationship could be established in this case. Increased numbers of compound cilia associated with decreased mucociliary transport have also been observed in regenerating mucosa of rabbits (Min, Kim et al. 1994).

1.7.2 Translocation/ transposition

In this abnormality, the central pair of microtubules appears to have been replaced by one of the peripheral microtubule doublets. It is commonly found in PCD patients and is specifically associated with a rigid grabbing motion, slight bending of the distal third of the cilia, a poor recovery stroke and incomplete effector stroke (Rossman et al. 1984). Functionally, these cilia are believed to retain about 10% normal motility. Transposition is thought to be caused by a shortage of nexin links and radial spokes (Van der Baan et al. 1987). In some cases of PCD, transposition is the only defect found (Sturgess et al. 1980). It was first described as a cause of PCD in two siblings with a chronic sinobronchial syndrome in whom this abnormality, thought to be due to a defective central sheath, was observed in both respiratory cilia and in the sperm of the male patient (Sturgess et al. 1980). Only a few of the cilia examined had two central tubules which extended a short distance from the cell surface.

1.7.3 Peripheral microtubular

Supernumery peripheral microtubules or missing peripheral microtubules may occur. If supernumerary, the extra microtubules are usually outside the normal axonemal arrangement as illustrated in Figure 1.4. Mircrotubular anomalies are common in, but not usually the cause of, PCD (Van der Baan et al. 1987). They are also thought to be acquired abnormalities and increased incidence is associated with acute and chronic disease (Carson, Collier et al. 1985; Carson, Collier et al. 1994), but their significance is unclear.

1.7.4 Central complex

Randall et al (1964) described a paralysed *Chlamydomonas* mutant which lacked the central pair of microtubules (Randall et al. 1964). Cilia lacking in the central pair have been described as an acquired defect in recurrent infections where only a few cilia are affected (Lungarella et al. 1983; Carson et al. 1985) and as a congenital defect where up to half the cilia are affected (Rossman et al. 1984; Tamalet et al. 2001). Tamalet et al (2001) studied partial ciliary defects in children with chronic or recurrent lower respiratory tract infections and found that children with central complex defects as the most common abnormality had the most severe symptoms of disease compared with children with partial dynein arm deficiency or peripheral microtublular defects (Tamalet et al. 2001). The central complex abnormalities were associated with a higher incidence of respiratory tract infections and extensive bronchiectasis. Present in such high number, these abnormalities were thought to be of congenital origin, but it was unknown why this particular abnormality should result in more severe disease.

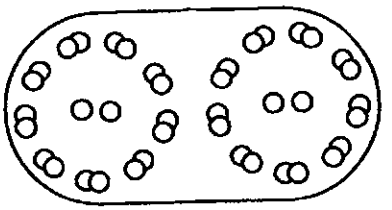
Afzelius (1976) postulated that normal visceral symmetry is determined by cilia in epithelial tissues of the early embryo. More precisely, it is the monocilia, or those of the

9+0 configuration (i.e. lacking in central tubules), which determine body laterality as their 'gyrating' motion results in leftward water flow and subsequent movement of the forming heart (Afzelius 1999). Respiratory cilia lacking the central pair of microtubules are also liable to move in this way, possibly impairing mucus transport.

1.7.4.1 Orientation

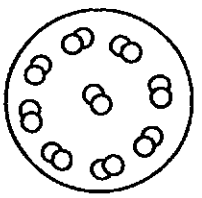
The orientation of the central pair of microtubules is also indicative of the direction of cilial beat. In order to achieve effective and efficient mucus transport adjacent cilia must be beating in the same direction. The importance of orientation is illustrated in certain cases of PCD where disorientation alone is the cause of disease (Rutland and de Iongh 1990; Rayner et al. 1996). Orientation has also been shown to be acquired secondary to infection. Rayner et al (1995) studied nasal mucociliary clearance along with ultrastructure and orientation of cilia in patients with upper respiratory tract inflammation caused by infection and found that disorientation without ultrastructural abnormalities was significantly associated with delayed mucus transport (Rayner et al. 1995).

Compound



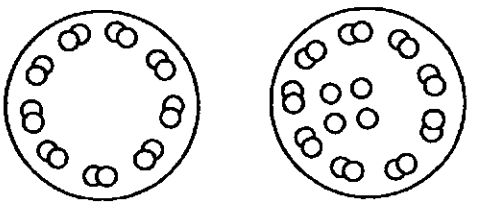
More than one axonemal arrangement within the same membrane.

Translocation



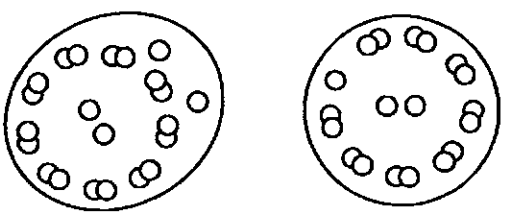
The central pair of microtubules is missing and appears to have been replaced by one of the outer microtubule doublets.

Central complex



The central pair may be absent or extra single tubules may appear within the centre of the axoneme.

Peripheral



Peripheral microtubule singlets or doublets may be missing or supernumery.

Figure 1.6 Cilial abnormalities found in the general population, including patients with PCD.

Early workers put forward the hypothesis that abnormal cilia are formed as a result of ciliary reconstruction after a deciliation caused by harmful agents (Friedmann and Bird 1971). It had already been confirmed however that deciliated cells decay and are replaced by immature cells which subsequently produce new cilia (Hilding and Hilding 1966). It is now thought that abnormal cilia arise from one of two pathways (Fonzi et al. 1982; Hagiwara et al. 2000). Ghadially (1975) thought that abnormal cilia, 'are either produced by a fusion of pre-existing cilia or arise as a result of multiple axial microtubule complexes entering a single large evagination of the plasma membrane'. Fonzi et al (1982) agreed with the former hypothesis (fusion of existing cilia) after studying the morphology of cilia before and after the instillation of elastase in rabbits. The bronchi of the instilled animals were examined by transmission electron microscopy at various time points following initial instillation in order to examine the ultrastucture of the cilia. Vesiculation of the ciliary membranes was seen after eight to fifteen days exposure to elastase. After fifteen to eighteen days the most common abnormality was absence of the membrane. It is thought this abnormality promotes fusion of several cilia and disorientation of the microtubules. It wasn't until sixty to ninety days after instillation that abnormalities such as compounds or cilia with missing or extra microtubules were observed. It was thus concluded that elastase instillation caused the formation of cilial abnormalities via the fusion of pre-existing cilia (Fonzi et al. 1982) (Figure 1.7). The second hypothesis for the origin of abnormal cilia is that they are formed during aberrant ciliogenesis (Figure 1.8). The process of ciliogenesis begins with duplication of the centrioles and their migration to the apex of the cell. The centrioles then form the basal bodies from which the ciliary shaft extends at the periphery of the luminal cell surface. Type 2, 'bulging' compound cilia are believed to be formed not by fusion of existing cilia but during the budding and elongation process of ciliogenesis when several centrioles migrate to existing cytoplasmic protrusions (Hagiwara et al. 2000).

Intracytoplasmic cilia, cilia within periciliary sheaths, and intracellular ciliated cysts are thought to arise from defective centriole migration (Hagiwara et al. 2000).

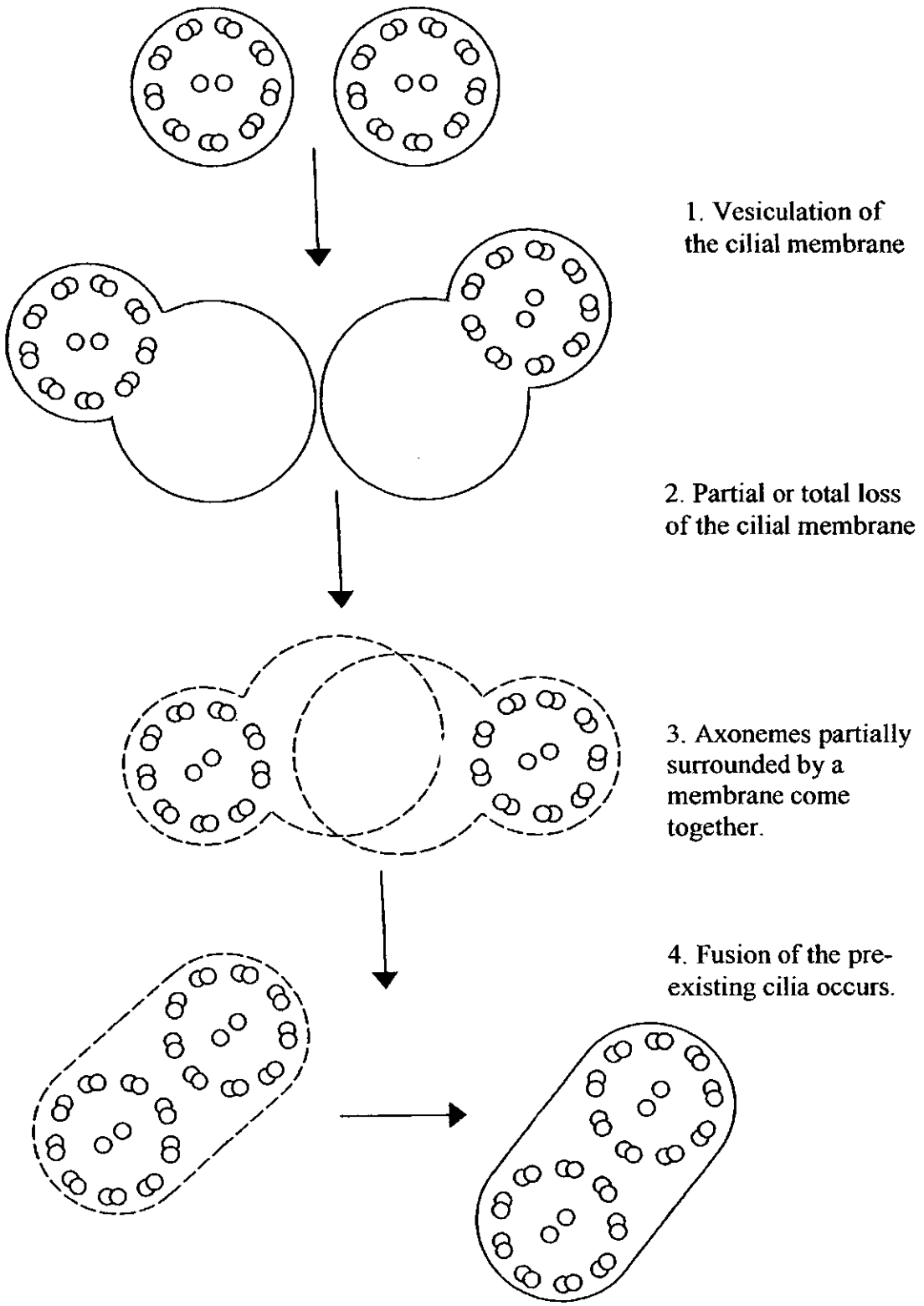


Figure 1.7 Sequence of events in the fusion theory for the formation of compound cilia

Adapted from (Fonzi et al., 1982)

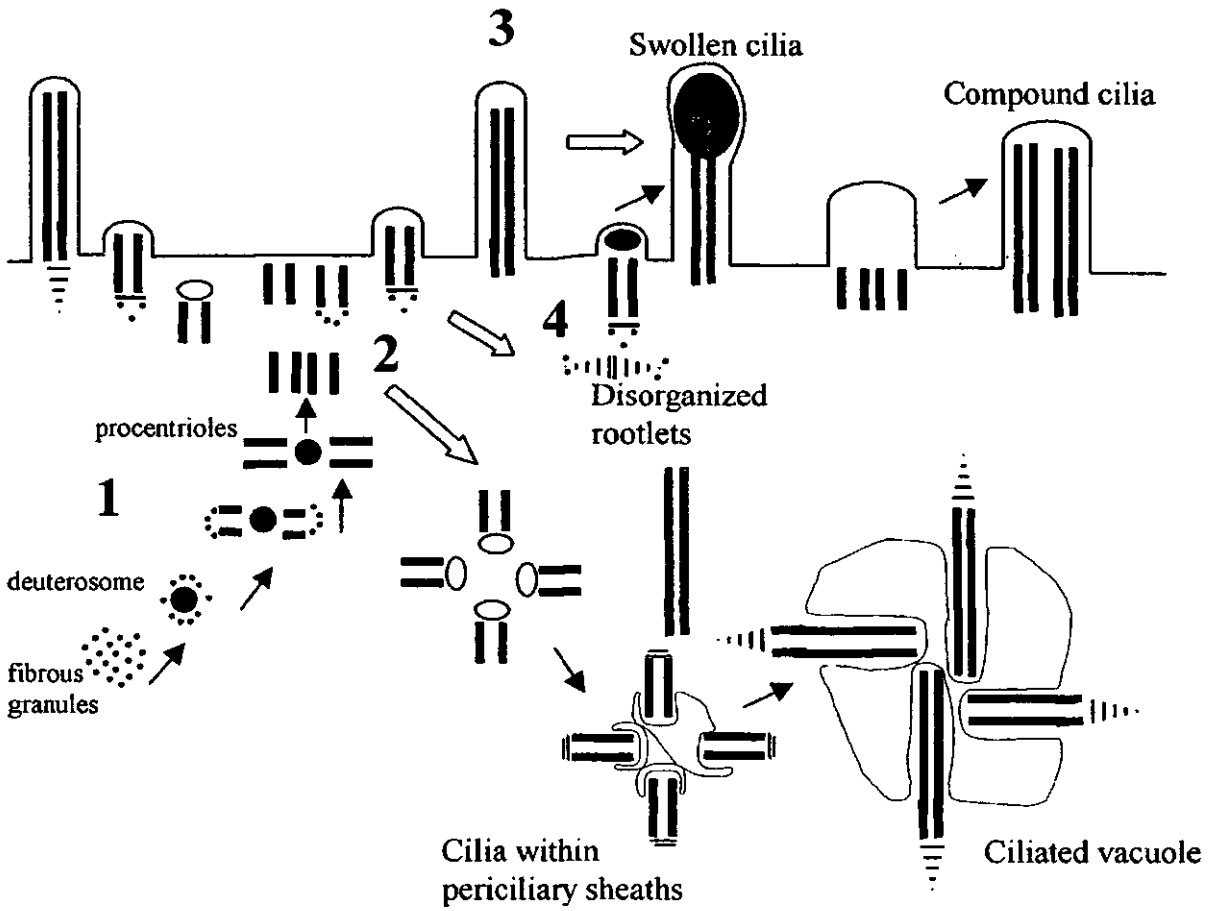


Figure 1.8 Ciliogenesis and formation of abnormal cilia

1 = duplication/ generation of centrioles

Fibrous granules form the fibrogranuloma complex with filamentous material. Deuterosomes then appear close to the aggregated fibrous granules and are the core of centriole formation. Procentrioles then develop into short cylinders containing microtubules.

2 = migration of centrioles to cell apex

The centrioles become the basal bodies from which the cilia elongate. Inhibition of migration results in the formation of cilia within periciliary sheaths and intracellular ciliated vacuoles.

3 = elongation of cilia

Swollen cilia and compound cilia may be formed at this point.

4 = formation of accessory structures of basal bodies

Determines arrangement of basal feet. They need to be orientated in the same direction (in order that the cilia beat in the direction as eachother).

The large block arrows indicate potential anomalous pathways resulting in the formation of abnormal cilia.

Adapted from (Hagiwara et al., 2000)

1.8 Incidence of cilial abnormalities

At least 40% of cilia in PCD patients usually lack the outer dynein arms and several studies have sought to determine the incidence of microtubular abnormalities in different subject groups. Healthy subjects appear to have less than 5% abnormal cilia. Some of these studies are summarised in Table 1.

Table 1.1 Summary of studies reporting incidence of cilial abnormalities in human health and disease.

Patients/ population studied	N	Type of sample	Was the study blinded?	Percentage abnormal cilia	Number of cilia counted per sample	p	Comments	Authors (year)
Lung diseases	35	Bronchial biopsy	Not stated		> 500	< 0.0001	Excluded compound cilia.	Fox and Bull (1983)
Smokers	27			3.0				
Non-smokers	8			2.4				
Ex-smokers	8			2.1				
Present smokers	10			3.6				
Lung cancer	19			3.4				
No lung cancer	13			2.4				
Chronic pulmonary infection	15			3.7				
No infection								
Retinitis pigmentosa	19			2.4				
Healthy controls	7	Nasal biopsy		7.4				
	5			2.4				
Cystic fibrosis	16	Nasal brushings	Yes	2.5	163 ± 104	< 0.01	Approx values only (read from bar chart).	Rutland et al (1983)
Bronchiectasis	15			6.0				
Kartagener's	15			27.0		< 0.001		
Healthy controls:								
Smokers	10			3.0				
Non-smokers	10			2.0		< 0.05		
Cystic fibrosis	5	Nasal curettage	Yes	6.2	≥ 200	< 0.0001	No differences within normal group or between normals and cystic fibrosis or rhinitis.	Rossman et al (1984)
PCD	9			31.5 – 95.5				
Chronic rhinitits	7			4.5				
Atopic non-smokers	4			3.1				
Asymptomatic smokers	8			2.8				
Non-atopic non-smokers	18			4.8				
PCD	29	Nasal biopsy	Not stated	Not given	20		PCDs had less nexin links, radial spokes, and more transpositions. Infection group had more transpositions than healthy group	Van der Baan et al (1995)
Chronic respiratory infection	71							
Healthy controls	23							
PCD	31	Nasal brushings.	Not stated	12.7	≥ 200	[No sig diff.]	Dynein arm analysis presented separately. No differences in incidence of compounds between PCD, bronchiectasis and controls.	De Iongh and Rutland (1995)
Bronchiectasis	20			4.5				
Healthy controls:								
Non-smokers	31			3.8				
Ex-smokers	20			4.0				
Smokers	11			2.8				

1.9 *Mucociliary interactions*

Effective mucociliary transport is dependent upon the optimal interaction between the cilia and overlying mucus. The integrity of the axoneme is crucial for normal ciliary beat pattern and frequency as described above. At the tip of the cilia there are three to seven short 'claws' which engage the overlying mucus during the effector stroke, possibly raising it a little from the cell surface and propelling it forward. MTR is also critically dependent on the viscoelastic properties of the mucus. MTR has been shown to be directly proportional to elasticity and indirectly proportional to viscosity (Sleigh et al., 1988). The depth of the periciliary fluid is of critical importance since if it were too deep, the tips of the cilia would not come into contact with the overlying mucus rendering CBF ineffective until the excess is removed/ absorbed. On the other hand, if the periciliary layer were too shallow, the cilia would encounter the mucus during the recovery as well as the effector stroke and become impeded.

Mucus is transported due to the co-ordination of many cilia and the resulting metachronal waves. Co-ordination of the cilia depends on simple mechanical recruitment and, in part, on the fluid surrounding each cilium because, 'if two cilia lie close enough together for the entrained fluid to overlap as they move, then these cilia will adjust their frequency and phase of beating so as to minimise this interference' (Sleigh et al., 1988).

CHAPTER TWO

Literature review and Aims

The mucociliary transport system is an important defence mechanism against respiratory infection. Efficient mucus transport and clearance of inhaled foreign particles is critically dependent upon the structural and functional integrity of the cilia and overlying mucus as described in the previous chapter. Reduction in the rate of mucus transport has been reported in intensive care patients and is likely to contribute to the increased risk of respiratory infection that these patients face. Nosocomial pneumonia is the most common infection in critically ill patients and the second most common hospital acquired infection overall.

2 Hospital Acquired Infection

There are at least 100, 000 cases of hospital acquired infection (HAI) a year in the UK and it has been estimated that five thousand deaths per year (1% of all deaths) occur as a direct result. HAIs could be a significant contributing factor in a further fifteen thousand deaths per year and it has been suggested that HAIs may cause more deaths than traffic accidents and suicides (Report for the House of Commons, 2000). In addition HAI's have been estimated to cost the health sector in England as much as £1bn each year. Research by the Central Public Health Laboratory Service (PHLS) and the London School of Hygiene and Tropical Medicine (LSHTM) carried out for the Department of Health between 1994 and 1995 showed that on average patients with HAIs spend two and a half times longer in hospital and cost £3000 more to treat (Plowman et al., 2000).

HAIs are defined as those which manifest seventy two hours or more after admission to hospital (i.e. not present or incubating before admission). Prevalence and incidence vary depending on the study population. In the UK, two prevalence surveys were carried out in 1980 (Meers et al., 1981) and 1994 (Kelsey et al., 2000). The urinary tract, surgical wound and lower respiratory tract were the most frequent sites of infection. The rate of respiratory tract infections appeared to rise from 21% in 1980 to 34% in 1994 although this may not be a true reflection since neither study surveyed onset of HAI post-discharge. The impact of HAI can be described in terms of the cost to the patient and to the NHS. HAIs add to the patients discomfort and may permanently affect their health in addition to stress caused to the patients and their relatives. The financial impact is variable depending on the patient population studied but is most commonly associated with increased length of stay. Other additional costs are incurred from the prolonged use of antibiotics and additional laboratory tests. Currie et al (1999) estimated that reductions in the incidence of HAI by 20%, 35%, and 50% across the UK could produce annual savings of £15.6 m; £29.3 m and £50 m respectively.

UK data are consistent with those in other parts of Europe and the USA. Around one in ten patients has an infection acquired in hospital and susceptibility varies with factors such as age, pre-existing condition, and treatment received. Intensive care patients are particularly at risk since they are usually the most ill. In the European Prevalence of Infection in Intensive Care (EPIC) study (Spencer 1994) and data from the USA's National Nosocomial Infections Surveillance System (NNIS) (Horan et al., 1986) incidence varied from seven to fifty four per one hundred patients.

2.1 Nosocomial Pneumonia

According to both the NNIS and EPIC, pneumonia is the second most common HAI overall and the most common infection in intensive care. The EPIC study collected data on 10,038 patients in ICUs from 17 different countries. ICU acquired infections accounted for 21% of infections, of which 47% were pneumonia and 18% other lower respiratory tract infections. In addition to being the most common infection in the ICU, pneumonia is also associated with the highest mortality and morbidity (Gross and Antwerpen 1983). One report states that approximately one quarter to one third of all patients who develop nosocomial pneumonia die, who would not die otherwise and published mortality rates range from 20% to over 70% with the median reported 42.5% (George 1995). Several pathogens cause nosocomial pneumonia but the greatest mortality rates are associated with *Pseudomonas aeruginosa* infection. The most commonly isolated pathogens are enterobacteriacae, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, though many cases have been found to be polymicrobial. However, the prevalence of different pathogens is thought to vary depending on the reliability of the culture used for identification (George 1995). Studies using sputum and tracheal aspirate cultures have been found to overestimate the prevalence of enterobacteriae due to the unreliability of the culture technique. Studies using more specific sources are thought to be more accurate and it appears that enterobacteriae are less prevalent than *Pseudomonas aeruginosa, Staphylococus aureus* and *Acinetobacter* species (George 1995). Other known pathogens to a lesser extent include the following: *S. pneumoniae*, *Haemophilus influenzae*, legionella species, aspergillus, cytomegalovirus, respiratory syncitial virus and occasionally influenza A.

Additional morbidity and cost are associated with nosocomial pneumonia and this includes an increased length of hospital stay. An increased length of stay is a likely outcome of infection and has been reported to vary from four to thirteen days. An average of 5.9 days has been stated by the Centers for Disease Control (CDC) (George 1995).

2.2 Risk factors for infection

Disturbance of the mucociliary transport system is likely to promote the retention of secretion in the respiratory tract thereby increasing the risk of nosocomial infection. Several factors associated with nosocomial pneumonia in ICU patients have been identified and part of the increased risk they present may be explained by the damage they cause to the mucociliary transport system. Some of these factors are as follows: duration of mechanical ventilation, humidification, anaesthesia, hyperoxia, previous smoking history, advanced age, and numerous therapeutic drugs. Combined, these factors may have a considerable negative impact on the mucociliary function in vulnerable ICU patients.

Mechanical ventilation

The link between respiratory devices and nosocomial pneumonia has been recognised for several decades (George 1995). Most cases of nosocomial pneumonia occur in patients who are tracheally intubated and/ or ventilated patients. Various studies report an increased risk for infection from three to twenty-one fold with tracheal intubation and ventilation. There is a daily risk of between 1 and 3% for developing ventilator associated pneumonia (VAP) (Ruiz-Santana et al., 1987; Langer et al., 1989). This risk is thought to change over time and almost half of all cases of VAP begin within the first four days of ventilation (Mehta and Niederman, 2003). Early onset VAP, which occurs within the first five days of hospitalisation, is caused by community organisms whereas late onset VAP, which occurs

after the first five days, is more likely to be due to antibiotic resistant organisms. The distinction between length of hospitalisation and length of ventilation is therefore critical from the treatment and prognosis perspective since late onset VAP is much less preventable and carries a higher mortality than early onset VAP (Mehta and Niederman, 2003).

Innoculation can occur as a result of aspiration, inhalation, or hematogenous dissemination. The most common mode is thought to be aspiration of upper airway secretions (Winn 1990; George 1995; Cooke and Watson 1996). Intubation predisposes to aspiration because the natural defence mechanisms are by-passed by the very presence of the endotracheal tube itself. The airway mucosa may also suffer mechanical damage and drying as a result of intubation (Levine and Niederman 1991). Lesions are caused directly by the tracheal tube (tracheobronchial toilet and tube movements) as well as by suctioning and fiberbronchoscopy. In addition, the tracheal tube, any other equipment for respiratory therapy and respiratory tract instrumentation can serve as reservoirs of bacterial growth. After just a few hours a biofilm may form on the lumen of the tracheal tube, and the source of contamination is usually from the patient's own secretions (Winn 1990; George 1995; Cooke and Watson 1996) .

Damage directly due to intubation has been assessed previously. For example, Sanada et al (1982) studied the effect of endotracheal intubation in mongrel dogs. Severe ciliary injury was observed immediately and three days following cuff deflation at extremely low pressure and for a short time period. The severity of injury correlated directly with the observed delay in mucociliary transport. The alterations in morphology and mucus transport were found to be reversible after two weeks. Alexopoulos et al (1984) studied the

effect of different grades of tracheal injury produced experimentally with a tracheal tube in pigs. After intubation or tracheotomy and ventilation for four hours, the mucociliary transport was measured *in vitro* and tracheal morphology studied using light microscopy and scanning and transmission electron microscopy. They observed a variable amount of damage, from none at all to patchy loss of a large proportion of cilia and a complete loss of cilia. In the absence of mucociliary transport, mucus clearance may be achieved by a compensatory mechanism such as coughing. The presence of an endotracheal tube inhibits this response as well as causing direct damage to the epithelium.

Studies by Konrad et al (1994, 1995) have demonstrated reduced mucociliary transport in ICU patients undergoing mechanical ventilation. Their first study established that bronchial mucus transport was impaired in ventilated ICU patients (Konrad et al., 1994). Bronchial mucus transport velocity (BTV) was measured by recording the movement of radiolabelled microspheres deposited at the distal end of the right and left main bronchus using a scintillation camera. This procedure was carried out within the first three days of mechanical ventilation and patients were examined daily for up to four days for the presence of pulmonary complications. BTV was impaired in all the patients and was significantly lower in those individuals who went on to develop retention of secretion and pneumonia. Konrad et al (1995) went on to investigate possible causative factors and carried out a similar study in which BTV was again measured in mechanically ventilated ICU patients. This time biopsy samples were taken for scanning and transmission electron microscopy. These measurements were also taken within three days of ventilation commencement. Patients with markedly depressed BTV were found to have significantly more cilia on their luminal surface than the patients with normal or only moderately impaired BTV and there was a correlation between the number of cilia and BTV. There

were slightly more abnormal cilia in the patients with the slowest BTV than in the patients with faster BTV (6.5 vs 9.3% respectively) but this difference was not statistically significant. There was no correlation between the incidence of abnormal cilia and BTV. The authors concluded that impaired mucus transport in intubated patients was associated with loss of cilia rather than ultrastructural abnormalities. However, these findings do have a number of important limitations. Firstly, the samples were all taken within three days of admission to the ICU. Thus only short term effects were studied, the loss of cilia probably being a direct result of intubation itself. The methodologies of the electron microscopy studies are also limited. The SEM investigation utilised a semi-quantitative technique and could be a subjective measurement. Two hundred cilia were counted in quantifying ultrastructural abnormalities but previous studies have shown that much larger numbers need to be studied in order to obtain an accurate result. Longer term studies are not evident from the literature and the first such study is presented in Chapter 6 of this thesis. The research presented in this thesis is a preliminary study which formed part of an ongoing investigation into the mucociliary transport system in longer term ICU patients.

2.2.1 Humidification

Intubation prevents normal heat and moisture exchange within the respiratory tract and it has been known for a long time that inadequate humidification and warming of the inspired gases causes morphological damage and drying of the mucosal lining, caused by the displacement of the isothermic saturation boundary (the point in the body at which inspired gas reaches core temperature and 100% relative humidity). Numerous studies have been carried out to determine the optimum temperature and humidity of inspired gases and a review of the substantial amount of literature relating humidification to mucus transport is

provided by Williams et al (1996). It is evident that both dry and excess moisture conditions decrease and eventually stop mucociliary transport. This effect has been shown to be reversible providing complete ciliostasis is avoided (Asmundsson and Kilburn 1970). Effects on mucus as well as cilia have been described (Tsuda, Noguchi et al., 1977). Drying of the mucosa leads to an increased viscosity of mucus, which is difficult for the cilia to transport, and insufficient depth of the periciliary layer causes the cessation of CBF by restricting the return stroke of the cilia. General histological changes which include loss of cilia and desquamation have also been described with a direct relationship with the duration of exposure (Chalon et al., 1972; Marfatia et al., 1975; Williams et al., 1996; Irlbeck 1998). The aim of the review article by Williams (1996) was to formulate a model for the relationship between airway mucosal function and the combination of the humidity of inspired gas and the duration of exposure. The optimal gas conditioning was found to be core temperature and 100% relative humidity. Conditions above or below this optimum would result in impaired mucus transport via the mechanisms outlined above, i.e. thickening of mucus, ciliostasis and histological damage. However, the model was only supported by evidence from conditions at or below the proposed optimum and only in the short term.

2.2.2 Anaesthesia

Duration of anaesthesia is a risk factor for the development of a respiratory infection in intensive care. This risk may be associated with the technique of administration and effects on lung mechanics but the role of anaesthesia *per se* and the anaesthetic and sedative agents themselves on mucociliary function has also been investigated. A number of studies have shown anaesthesia to impair mucociliary transport in both human and animal models. A summary of this data is shown in Table 2.1. While direct comparison between the

studies is limited by the different models and methods of assessment used it is clear that anaesthesia is detrimental to mucociliary transport. Most studies show a clear depressant effect of anaesthesia upon mucociliary transport, with the exception of the studies by Konrad et al (1992, 1997). They measured BTV by viewing the movement of radiolabelled microspheres with a scintillation camera, measuring BTV preoperatively and postoperatively. BTV was not influenced by general anaesthesia with midazolam, fentanyl, pancuronium and O_2:N_2O nor by that with isoflurane, fentanyl, vecuronium, O_2:N_2O (Konrad et al., 1992; Konrad et al., 1997). However, in contrast, a later study of theirs (Konrad et al., 1998), demonstrated a depression of mucociliary transport from 9.7 to 4.9 mm/ min in the right bronchus and from 11.3 mm/ min to 5.3 mm/ min in the left bronchus during total intravenous anaesthesia with propofol, alfentanil and vecuronium (TIVA).

Table 2.1 Summary of data from studies investigating the effect of anaesthesia upon mucociliary transport.

Anaesthetic(s)	Model	Method of assessing mucociliary transport	Effect of anaesthetic on mucociliary transport	Authors
Pentobarbital, thiomylal	Sheep	Movement of Teflon discs placed on the tracheal mucosa was measured using a cine-bronchofiberscopic technique to determine tracheal mucus velocity (TMV).	TMV was significantly reduced from control values of 17.3 mm/ min to 11.1 mm/ min during anaesthesia.	Landa et al., 1975.
Halothane, enflurane	Dog	Movement of radioactive droplets in trachea measured with external scintillation counters.	Decrease in tracheal mucociliary flow of 59 – 77% of control values during exposure to 1.2 MAC halothane and enflurane.	Forbes and Horrigan., 1977.
Halothane, enflurane, NLA, epidural	Human	Coloured transport indicator test measured mucociliary function in the nose and pharynx.	Halothane and enflurane were associated with a significant reduction in mucociliary activity.	Cavaliere et al.., 1983
Halothane	Rabbit	Mucociliary activity measured with a photoelectric technique.	Temporary increase of 39% in activity after administration, followed by decrease of up to 19.6% after 60 minutes exposure.	Cervin et al.., 1995
Halothane, isoflurane, desflurane	Rabbit	As above.	Administration of 1.0 MAC of each anaesthetic caused a temporary increase in mucociliary activity of up to 23.7%, the responses to each also being biphasic.	Cervin and Lindberg., 1998
Midazolam, fentanyl, pancuronium and O_2:N_2O Isoflurane, fentanyl, vecuronium, O_2:N_2O	Human	Movement of radiolabelled microspheres measured with a scintillation camera to determine bronchial mucus transport velocity (BTV).	No effect.	Konrad et al.., 1992; 1997
Propofol, alfentanil, vecuronium (TIVA)	Human	As above.	Depression of mucociliary transport from 9.7 to 4.9 mm/ min in right bronchus and from 11.3 to 5.3 mm/ min in left bronchus.	Konrad et al.., 1998.
Temazepam	Human	Tracheobronchial clearance assessed from changes in radioactivity of the lungs after inhalation of labelled particles.	Reduction in tracheobronchial clearance of 22% after temazepam compared with placebo during the first three hours following drug ingestion.	Hasani et al.., 1992.

Once again attention is now turned to the factors responsible for efficient mucus transport. Anaesthesia has the potential to disturb both the cilia and mucus. Pizov et al (1992) suggest that the impairment of mucus transport associated with inhalational anaesthesia may in part be due to a decrease in the volume and increase in viscosity of the mucus. Using an electrophysiologic technique, they investigated the effect of halothane on ion transport in canine tracheal epithelia *in vitro* (Pizov et al., 1992). Halothane was found to inhibit ion transport in a reversible dose-dependent manner, which is likely to impair fluid secretion and increase viscosity, which would impair mucus transport. However, more direct evidence does not support the hypothesis that anaesthesia alters the properties of mucus. Rubin et al (1990) collected samples of mucus from the endotracheal tubes of patients undergoing elective surgery during general anaesthesia. Rigidity, viscoelasticity, spinnability, and the percentage solid composition of the specimens was measured in addition to the transport of the collected mucus across the mucus-depleted frog palate. There were no significant differences in any of these properties between these samples and those collected from awake volunteers using the bronchoscopy brush collection technique (Rubin et al., 1990).

Conversely, numerous studies are suggestive of a direct effect of anaesthesia on the function of cilia. Early studies in *Tetrahymena pyriformis* demonstrated a depressant effect of several anaesthetic agents on swimming velocity (Nunn et al., 1974). Manawadu et al (1978, 1979) semi quantitatively assessed the effect of several anaesthetics at different concentrations on ciliary activity in ferret tracheal rings. A reversible effect of licocaine, procaine, chloroprocaine, and short-term exposure to halothane on ciliary activity was noted, but ciliostasis induced by bupivacaine was irreversible and longer term exposure (4 days) to halothane was found to cause death of ciliary cells (Manawadu et al., 1979).

Table 2.2 Summary of data from studies investigating the effect of anaesthetic agents on cilia beat frequency (CBF).

Anaesthetic (s)	Model	Method of CBF measurement	Effect of anaesthetic on CBF	Exposure of tissue to drug – *in vitro* or *in vivo*?	Authors
Halothane, enflurane, nitrous oxide.	Rabbit tracheal segments.	Photoelectric.	Halothane and enflurane caused a dose dependent depression. No effect of Nitrous oxide.	*In vitro*	Lee and Park, 1980
Lidocaine.	Human nasal brushings.	Photoelectric.	Concentrations greater than 2×10^{-2} g/ ml caused a dose dependent decrease.	*In vitro*	Rutland et al., 1981
Morphine, atropine Lidocaine,	Human nasal and tracheal brushings.	Photoelectric.	Premedication with morphine and atropine, and local anaesthesia with lidocaine significantly reduced CBF.	*In vivo*	Roth et al., 1991
Halothane.	Human nasal brushings.	Transmitted light technique.	Significant decrease in CBF after 2 hr exposure. Also increase in variability of measurements. Significant decrease in CBF after 4 hr exposure to 2.25% (3 MAC) halothane.	*In vitro*	Gyi et al., 1994 Raphael et al., 1996
Fentanyl. Sufentanyl.	Human nasal brushings.	Photoelectric.	Significant decrease in CBF after 1 hr exposure to 10 µg/ ml^{-1}.	*In vitro*	Rusznak et al., 1994
Enflurane.	Human nasal brushings.	Transmitted light technique.	Significant decrease in CBF after 4 hr exposure to 5% (3 MAC) enflurane.	*In vitro*	Raphael et al., 1996
Isoflurane.	Human nasal brushings.	Transmitted light technique.	Significant decrease in CBF after 4 hr exposure to 3.6% (3 MAC) isoflurane.	*In vitro*	Raphael et al., 1996
Morphine.	Human nasal brushings.	Transmitted light technique.	No significant effect after 4 hr exposure to 4 µmol/ $litre^{-1}$	*In vitro*	Selwyn et al., 1996
Propofol.	Human nasal brushings. Cultured rat tracheal tissue.	Transmitted light technique.	No significant change after 90 minutes exposure to 70 µm propofol. Significant dose dependent increase in CBF.	*In vitro* *In vitro*	Hann et al., 1998 Shirakami et al., 2000
Midazolam.	Human nasal brushings.	Transmitted light technique.	No significant change after 90 minutes exposure to µ20 m midazolam.	*In vitro*	Hann et al., 1998

Following these early studies, there has been an abundance of literature concerning the effects of different anaesthetic agents upon respiratory cilia in animal and in humans. A summary of this data is presented in Table 2.2. It is clear that most of the anaesthetic agents investigated have a deleterious effect upon CBF, which probably contributes to the impairment of mucociliary transport observed during anaesthesia. Much of the work in the 1990s was carried out by Selwyn et al (1996) who designed and built a perfusion chamber specifically for investigating the effects of highly volatile inhalational anaesthetic agents. The chamber was designed in order to allow samples of epithelium to be maintained for several hours in a stable and controlled environment, permitting repeated measurements of CBF during exposure (Selwyn., et al. 1996). They went on to investigate the effects of halothane, enflurane, and isoflurane on CBF using this system and found a dose and time dependent decrease in CBF with all three agents (Raphael., et al. 1996). Further work involved repeating these exposures with similar results, but then observing the recovery of CBF after a washout period of 90 minutes for halothane and 60 minutes for enflurane and isoflurane. In contrast to this depressant effect of inhalational anaesthetics on CBF, and despite being shown to decrease MTR, morphine had no such effect on CBF. The effects of two intravenous anaesthetic agents have been investigated with another negative result. Hann et al (1996) exposed nasal turbinate explants to supra-clinical concentrations of midazolam and propofol for 90 minutes and found no significant difference in CBF between exposed explants and controls (Hann et al., 1998). Interestingly, Shirakami et al (2000) found propofol to have a stimulatory effect on CBF in cultured rat tissue. Hann et al (1998) carried out more experiments with longer exposure periods. Still no effect on CBF was recorded. However, an unexpected effect of these agents on cilia survival was observed. This was formally investigated by the same group by noting the presence or absence of cilia after 24, 48 and 72 hours exposure to varying concentrations of drug. The

observation with propofol was not confirmed, but midazolam was found to have a time and dose-dependent effect on cilia survival, with only three of sixteen explants surviving after 48 hours exposure at the highest concentrations. Abundance of cilia in the respiratory tract is likely to influence the efficiency of mucus transport but these previous observations were largely subjective since they were not highly quantitative and this limits the findings.

2.2.3 Hyperoxia

It is common practice to administer elevated fraction of inspired oxygen to critically ill patients. Breathing an increased fraction of oxygen would appear however to be deleterious to the mucociliary transport system. Decreased mucus transport rates resulting from the breathing of high oxygen concentrations have been demonstrated in animal models and in humans. For example, Laurenzi et al (1968) (Laurenzi et al., 1968)demonstrated the adverse effect of any deviation from ambient oxygen high or low on tracheal mucus transport in cats by direct observation of particles deposited in the trachea. Breathing concentrations of 10, 40, and 100% oxygen for twenty minutes decreased TMV by 43, 23, and 37% respectively. The effects were reversible after five minutes breathing compressed air. A study using mongrel dogs ventilated through a divided tube enabled the comparison of clearance rates between one lung which received air and the other lung which received 100% oxygen. Clearance of tantalum powder by means of serial x-rays was found to range from 24 – 48 hours in air ventilated lungs and from 48 – 72 hours in the 100% oxygen ventilated lungs (Wolfe et al., 1972). In humans, measurements of TMV made by following the movement of Teflon discs placed on the trachea, were found to be decreased after breathing 90-95% oxygen for three hours (Sackner et al., 1975). It is clear that there is a deleterious effect of high concentrations of oxygen on the mucociliary transport system.

Many studies have investigated the effect of high oxygen concentrations on the histology of the respiratory mucosa (Boat 1979; Obara et al., 1979; Barnes et al., 1983; Konradova et al., 1987; Konradova et al., 1988; Nikula and Wilson 1990; Nikula et al., 1991). Obara et al (1979) described morphological changes in the bronchial and bronchiolar epithelium as observed by SEM in mice exposed to 95 – 100% oxygen for four days (Obara et al., 1979). The exposure was associated with denudation and truncation of the cilia, bleb formation, and desquamation in the bronchus and flattening of the luminal surface in the bronchiole. These alterations were found to persist for at least two weeks following cessation of exposure. Exposure is thought to cause an increase in the number of goblet cells and increase in mucus secretion as well as loss of cilia (Weisman and Sadé 1979; Konradova et al., 1988). There is, however, a lack of quantitative work in this area.

The adverse effect of elevated fraction of inspired oxygen on ciliary activity has also been studied. It appears that there is an initial increase in ciliary activity after short term exposure, but a decrease after long term exposure. Barnes et al (1983) investigated the effects of elevated PO_2 on hamster tracheal explants *in vitro*. In addition to morphological observations made using light microscopy and scanning electron microscopy, relative ciliary activity was defined by the percentage of the epithelial layer remaining intact times the vigour of ciliary beating (on an arbitrary scale). This study was limited by a subjective scoring system but showed that ciliary activity decreased by 25% following exposure to 60% oxygen for 120 hours, by 50% following exposure to 95% oxygen within 72 hours and ciliostasis after 144 hours exposure to 95% oxygen. Exposure to 20% and 40% oxygen for 144 hours did not affect ciliary activity. The morphological studies confirmed the loss of cilia and increased mucus secretion after three days exposure to 95% oxygen. Focal flattening of the epithelium was also noted as in previous studies. The effect was less

pronounced following exposure to 60% oxygen and was not apparent after exposure to 20% and 40% oxygen. (Barnes et al., 1983). More recently, a short term investigation into the effect of oxygen on CBF in human nasal turbinate epithelium was carried out (Stanek et al., 1998). Samples of respiratory epithelium were exposed to different concentrations of oxygen for up to 240 minutes. There was a dose and time dependent accelerating effect on CBF as measured by videotaping images of beating cilia and direct counting during slow motion play back. The accelerating effect began to reverse after 240 minutes exposure to 95% oxygen.

2.2.4 Smoking

Smoking is the main (preventable) risk factor for chronic bronchitis and obstructive airways disease. Although there is conflicting evidence in the literature, the bulk of it supports an increased rate and severity of respiratory infection in smokers (Marcy and Merrill 1987). Not only that, the incidence and severity of symptoms has been shown to be increased in children exposed to passive smoke from their parents (Gryczynska et al., 1999). Furthermore, this effect appears to be dose dependent with a lower incidence of bronchitis and pneumonia in children of one smoking parent compared to those of two smoking parents.

In hospitalised patients, smoking is associated with an increased risk of developing a postoperative infection (Dilworth and White, 1992; Beckers and Camu, 1991; Pearce and Jones, 1984). This risk is likely to be related to the fact the respiratory tract of smokers is frequently already colonised by bacteria (Irwin et al., 1982). This is probably caused by impairment of the mucociliary transport system (Gamsu et al., 1979), a subject which has been heavily studied in smokers and has caused some controversy over the years. Different methods of assessing mucociliary clearance make direct comparisons within the literature

difficult and mucociliary clearance has been shown to be increased, decreased and remaining the same when compared to healthy non-smokers (Wanner, 1985). However, from experiments in different animal species and studies in humans, it is generally believed that long term smoking has an adverse effect on mucociliary clearance. A summary of findings of studies investigating the effect of cigarette smoking upon mucociliary clearance and CBF is presented in Table 2.3. Mucus clearance also appears to be repressed in ex-smokers (Mortensen et al., 1994) and Goodman et al (1978) demonstrated that the inhibition of mucus transport may be subject to inter-individual variation with some, but not others, being affected at all (Goodman et al., 1978). This reduction in mucociliary transport may be reversible after cessation of smoking for more than three months but not after only a week (Camner et al., 1973).

Table 2.3 Summary of data from studies on the effect of cigarette smoking on mucociliary clearance and CBF.

Model	Mucociliary clearance	CBF	Notes	Authors
Rabbit trachea *in vitro*, cat trachea *in vivo*.		Exposure to 1 ml and 10 ml puffs of tobacco smoke required 73 puffs and 71 puffs to produce ciliostasis in the rabbit and cat trachea respectively.	No measurement of CBF - cilia were observed through the light microscope and exposure continued until ciliostasis occurred.	Dalhamn, 1970
Maxillary sinus of rabbits *in vivo*.		Cigarette smoke was found to accelerate mucociliary wave frequency.		Hybbinnette, 1982
Asymptomatic smokers and non-smokers.	50% and 25% retention times in smokers up to 90% greater than in non-smokers, although 6 of the 8 smokers had 75% retention times comparable to those in the non-smokers.		Mucus transport measured by using a gamma camera to follow movement of inhaled radioactive particles. Quantitated as time taken from deposition to reach 75%, 50% and 25% retention levels.	Foster et al., 1985
Healthy smokers and non-smokers.	Nasal mucociliary clearance of saccharin found to take significantly longer in the smokers.	No significant difference between the smokers and non-smokers.	There were no significant differences in mean CBF or nasal mucociliary clearance immediately after 10 healthy non-smokers had smoked 2 cigarettes each.	Stanley et al., 1986
Patients with chronic obstructive bronchitis and healthy non-smokers.	Rate of mucociliary clearance in smokers reduced to 65% that found in healthy non-smokers.		Aerosol scintigraphy of the lungs used to assess mucociliary transport.	Iun et al., 1991
Patients undergoing major abdominal or thoracic surgery with postoperative mechanical ventilation.	Smokers had a significantly slower bronchial mucus transport velocity than the non-smokers.		Bronchial mucus transport velocity determined by viewing radiolabelled microspheres with a scintillation camera.	Konrad et al., 1993
Nasal brushings from patients with middle ear disease, age and sex matched controls.		Nasal CBF in tobacco smoke exposed patients was significantly less than that in the non-smoke exposed individuals.		Agius et al., 1998

As already stated, smokers are known to be at risk of developing a respiratory infection in the post-operative period and in intensive care (Dilworth and White 1992). A study by Konrad et al (1993) investigated bronchial mucus transport rates in smokers and non-smokers during anaesthesia, having undergone major upper abdominal or thoracic surgery with postoperative mechanical ventilation. Bronchial mucus transport velocity was determined by recording the movement of a radiolabelled bolus deposited during bronchoscopy using a scintillation camera. Smokers were found to have a significantly slower bronchial mucus transport velocity than non-smokers (in the left bronchus: median 1.3mm/ min v 9.7mm/ min in smokers and non-smokers respectively). Pulmonary complications (retention of secretion defined atelactasis requiring suctioning and pneumonia) occurred in four of twelve smokers and just one of twenty two non-smokers (Konrad et al., 1993).

Factors which contribute to efficient mucociliary transport include the mucus and structure and function of the cilia. Mucus hypersecretion is often found in smokers, particularly in chronic bronchitics. However, cilia beat frequency (CBF) is thought to the most important determinant of mucus transport rate (MTR) with small changes in CBF leading to large changes in MTR (Duchateau et al., 1985). Numerous studies have investigated the effect of cigarette smoking on CBF both *in vitro* and *in vivo*, as shown in Table 2.3. Experiments *in vitro* show that tobacco smoke has a clear ciliotoxic effect with exposure of explants to smoke resulting in ciliostasis and even demonstrate the dose-dependent nature of the response (Kawada et al., 1991). However, *in vitro* measurements of CBF in smokers compared to non-smokers yield conflicting results (Table 2.3).

Histological changes in the respiratory epithelium due to short term tobacco smoke exposure have not been carried out in humans, but enlargement of intercellular spaces, bulging into the lumen of apical portion of cells, and swelling of the cilia have been described in foetal rabbit tracheas exposed to puffs of smoke and then immediately fixed for TEM (Davies and Kistler 1975). Several early and more recent human studies in smokers and non-smokers have been carried out and the consensus is that pathological changes are present in smokers that are not as apparent in non-smokers. Animal models confirm these findings (Chalon et al., 1975; Ebert and Terracio 1975; Auerbach et al., 1979; Coggins et al., 1980; Riise et al., 1992). Destruction of epithelial cells, squamous metaplasia, denudation of cilia and goblet cell hyperplasia have all been described in association with chronic cigarette smoking.

Although histological changes to the respiratory epithelium in relation to smoking have been extensively described, there is little research that focuses entirely on the cilia themselves. The ultrastructural integrity of the cilia is vital to the efficiency of the mucociliary transport system as most dramatically illustrated by the congenital condition Kartagener's syndrome (see Chapter 1). Cilial abnormalities have been shown to be associated with chronic bronchitis. For example, in a study by Lungarella et al (1983) cilial ultrastructure was studied in bronchial biopsies from patients with chronic bronchitis and non-smoking controls. There was a significantly higher incidence (8 – 28%) of abnormalities in the patients with chronic bronchitis compared with the controls (0 – 6%). However, studies that have compared cilial abnormalities in *asymptomatic* smokers and non-smokers have yielded conflicting results. For example, Rossman et al (1983) and de Iongh (1995) studied cilial abnormalities in nasal brushings from a range of patients including asymptomatic smokers and non-smokers between which no difference was

observed (Rossman et al., 1983; De Iongh and Rutland 1989). In contrast, work by Rutland et al (1983) and Fox et al (1983) who again studied cilial ultrastructure in nasal brushings found there to be significantly more abnormal cilia in smokers than in non-smokers (Fox et al., 1983; Rutland et al., 1983). Although the results of these studies conflict, they have in common the primary aim being to compare several different groups of patients, the focus being on those with recurrent respiratory infections or a congenital disorder, with few healthy patients to compare them with. Of particular interest is a study by Verra et al (1995), an investigation of cilial abnormalities in the bronchial epithelium of smokers, ex-smokers, and non-smokers. Not only was a higher incidence of abnormal cilia found in the smokers when compared to non-smokers, but also in the ex-smokers compared to the non-smokers. However the patients in this study were known to suffer from chronic sputum production and there was only a small number of healthy controls. Verra's study was also severely limited by the lack of a blinding protocol when screening for abnormal cilia (Verra, Escudier et al. 1995).

2.2.5 Age

Elderly patients are more at risk of nosocomial infection than their younger counterparts (George 1995; Kelsey et al., 2000). Mucociliary transport has been shown to decline with advancing age (Puchelle et al., 1979) so it is possible that this contributes to the risk. Little is currently known about underlying mechanism for this decline and an increased proportion of abnormal cilia may be a partial explanation (Ho et al., 2001).

2.2.6 Drugs

There have been several investigations into the effect of different drugs on mucociliary clearance, which are of relevance to patients receiving intensive care. In addition to anaesthetic and sedative agents commonly used in the ICU (Table 2.1), examples of others studied include atropine, cortisone, beta-blockers, beta-mimetics, theophylline and mucolytics. Cortisone, beta-mimetics and theophylline have all been shown to have a stimulating effect on mucociliary clearance (Table 2.4). In contrast, a deleterious effect has been demonstrated with beta-blockers and studies concerning atropine and mucolytics have yielded conflicting results (Konrad 1995).

Table 2.4 Summary of data from studies investigating the effect of drugs used in intensive care upon the mucociliary transport system.

Drug	Mucociliary clearance	CBF	Authors
Atropine	Mucociliary transport measured before and 10 min after i.v infusion of atropine in dogs. Mucociliary transport rate increased by 30%.	Nasal brushings taken before and 20 min following i.v. injection of atropine in patients with otosclerosis. CBF decreased by 25%.	Chopra 1978 Centanni et al., 1998.
Cortisone	Significantly increased tracheal mucociliary transport rate in pigeons.		Kai et al., 1990.
Theophylline	Theophylline induced a significant improvement in bronchial mucus transport velocity in ventilated ICU patients but was associated with severe tachycardia.		Konrad et al., 1994.
Salbutamol		Cultured human bronchial epithelial cells exposed - 10^{-4} M salbutamol caused a transient but significant increase in CBF.	Devalia et al., 1992.

2.3 Aims

There is at present a lack of information concerning the mucociliary transport system in long term critically ill patients. The only studies on intensive care patients thus far have been carried out within three days of admission (Konrad et al., 1994; 1995). The length of stay on the ICU varies considerably but with each day there is a risk of developing a hospital acquired infection such as pneumonia. The primary aim of this study was to gain a greater understanding of the mucociliary transport system in critically ill patients by assessment of its structure and function in patients beyond three days admission to the ICU.

Several risk factors for the development of nosocomial infection have already been identified and those thought to affect the mucociliary transport system have been described in this context above. In order to further elucidate the contribution and mechanism of action of two known risk factors, additional aims of the study were to investigate the effect of cigarette smoking and specific anaesthetic agents on respiratory cilia.

Hypotheses

1. The mucociliary transport system of critically ill patients declines with increasing length of stay in intensive care.
2. The intravenous anaesthetic agents midazolam and propofol have a deleterious effect upon cilia survival *in vitro*.
3. Halothane induces detrimental changes to cilia beat form in vitro, in addition to its effect upon cilia beat frequency.
4. There is a higher incidence of cilial abnormalities in asymptomatic smokers than in healthy non-smokers.

In order to test these hypotheses the specific aims were as follows:

- Develop specific, robust and non-subjective techniques for the assessment of cilial abnormalities (transmission electron microscopy) and abundance (scanning electron microscopy/ image analysis).
- Assess the incidence of cilial abnormalities in ICU patients beyond three days admission.
- Incubate rat trachea with midazolam and propofol and assess cilia survival by means of image analysis.
- Use high speed digital video to investigate the effect of halothane upon cilia beat form as well as frequency.
- Determine the incidence of cilial abnormalities in brushings of tracheal epithelium taken from asymptomatic smokers and healthy non-smokers.

Together, the studies scrutinizing the risks associated the anaesthesia and smoking along with a descriptive investigation of the natural history of the mucociliary apparatus in ICU patients may have the potential to contribute to therapeutic decisions.

CHAPTER THREE

Image analysis for quantification of cilia survival

3 Abstract

Many studies concerning cilia survival rely on subjective, semi-quantitative methods by which to assess the amount of cilia in a sample of respiratory epithelium. In order to investigate the effect of two anaesthetic agents on cilia survival, a semi-automatic, highly quantitative image analysis technique was developed and tested by comparing it with another quantitative technique that was subjective and laborious. The aim of this experiment was to test the accuracy of the semi-automated technique. Scanning electron microscope (SEM) images of rat tracheal epithelium were digitally captured and analysed using both a customised script written for the Quantimet-570 (Q-570) image analyser (Cambridge Instruments, England) and PC based software Image Tool. The Quantimet-570 technique compared well with Image Tool ($r = 0.89$, $p<0.001$). There was a statistically significant difference between the two methods, the Q-570 measuring approximately 5% less cilial coverage than Image Tool, ($p< 0.01$, paired t-test). However, there was no correlation between the size of measurement and the amount by which they differed ($r= 0.17$, $p= 0.42$). Thus, the use of Q-570 was a reliable technique, semi-automatic, and required little user intervention ensuring minimal subjectivity.

3.1 Introduction

One of the aims of this programme of research was to study the effect of two anaesthetic agents upon cilia survival. The respiratory epithelium of the rat trachea is well characterised and has been described in SEM studies many times in the literature (Alexander et al., 1975; Jeffery and Reid 1975; Popp and Martin 1984; Wilson et al., 1984; Souma 1987). The trachea is lined by a pseudostratified epithelium containing ciliated cells interspersed with goblet cells, which may also posses microvilli. Scanning electron microscopy reveals the surface topography and distribution of cells whereas transmission electron microscopy can identify the presence of other cell types beneath the cilia.

In order to evaluate cilia survival, a means of measurement was required. Image analysis is a means of obtaining objective quantitative (i.e. numerical) data from an image, preferable to qualitative descriptions which are more likely to be subjective and less reproducible. Image analysis can be carried out manually by utilising morphometric and stereologic techniques. The terms morphometry and stereology are often used synonymously to describe the measurement of geometrical quantities of an object such as numbers, length, profile area, surface area, and volume. Morphometry is an established mathematical process by which images are systematically processed in order to obtain information about the morphology (i.e. measurement of shape, surface area) of target objects within the image. Stereology is essentially the process of obtaining three dimensional information from a two dimensional image. This is generally achieved by point counting using a specific stereological probe. A stereological probe consists of a geometrical entity such as an array of points, lines, or cycloids etc. A set of counting rules determine what actions can occur with the interaction, e.g. intersections of the probe with the structure of interest. However, the researcher has to make several judgements in this process including, for example, whether the structure is in or out of focus, in or outside a particular boundary

(Glaser and Glaser, 2000). Several techniques for microscopical quantification of ciliated cells have been described previously. For example, Jeffery and Reid (1975) carried out a quantitative and electron microscopic study to determine the distribution and frequency of each cell type in rat airway epithelium. This study (Jeffery and Reid 1975) utilised transmission electron microscopy and involved manual differentiation and counting of different cell types. From these counts a percentage of each cell type was then calculated and the percentage of ciliated cells in the upper and lower trachea was found to be 17% and 33% respectively. This technique would have been very labour intensive and limited in value since it may be highly dependent upon the correct orientation of the tissue. Other light and transmission electron microscopy (TEM) studies have also quantified cells by means of stereological techniques which involve cell and point-counting procedures (Wilson et al., 1984; Lemos et al., 1994). For example, Wilson et al (1984) carried out a quantitative study of tracheobronchial epithelium in the bonnet monkey. Entire sections viewed with the TEM were photographed and enlarged after which they formed a montage. Point counts were carried out using a clear plastic square grid overlay which was moved a random amount along the montage until the entire image had been covered (Wilson et al., 1984). Apart from being a time consuming method, the numbers of cells counted is likely to be influenced by their size and shape.

Unlike TEM, scanning electron microscopy (SEM) can be used to visualise the entire surface of a sample at one time. SEM is therefore more appropriate for investigations of surface topography and distribution of cells. Scanning electron microscopy has previously been used to describe the surface topography and distribution of cell types in respiratory epithelium (Alexander et al., 1975; Popp and Martin 1984; Souma 1987). However, these studies tend to be descriptive or involve manual counting of cells. Studies on cilia survival also often incorporate a semi-quantitative or descriptive method which is subject to

investigator error (Weisman and Sadé 1979; Konrad et al., 1995). For example, Weisman et al (1979) studied the effects of environmental carbon dioxide, oxygen and pH on the growth of respiratory epithelium *in vitro* using explants of rabbit trachea. The proportion of the surface area covered by cilia was estimated in sections by assignment of arbitrary units with 0 representing no cilial coverage of the epithelium and 4 representing complete cilial coverage. SEM was incorporated but in a descriptive application only (Weisman and Sadé 1979). The SEM analysis in Konrad's study (1995) of intubated patients also utilized semi-quantitative, arbitrary cut-offs. The percentage of the surface area covered by cilia was divided into three categories: ciliated area greater than 75%, between 75 and 50%, and less than 50% (Konrad et al., 1995). This represents a very crude and subjective estimate of cilia coverage not sufficient for quantifying cilia survival in controlled experiments.

Computer assisted image analysis is becoming established as a way of quantifying cells and tissue. Zahm et al (1990) used an image analysis method to quantify the amount of actively beating cilia which involved subtracting two successive digitized images and subsequent filtering and binary transformation of the resulting image. Using this technique they were able to quantify the percentage of surface area covered by ciliated cells (Zahm, Lamiot et al., 1990). Image analysis may also be used to quantify ciliated cells that have been immunologically stained using a monoclonal antibody (Clark et al., 1995; Ostrowski, Randell et al., 1995).

In order to evaluate cilia survival in a quantitative non-subjective manner, an image analysis tool was required. The aim of this part of the study was to develop and test an image analysis technique by which to quantify cilial abundance in terms of percentage surface area of the epithelium covered. This system was to be used in experiments investigating the effects of anaesthetic agents upon cilia survival. The percentage

suface area of an SEM image covered by cilia could be estimated in several ways as described above. In order to maximise reproducibility and objectiveness in the measurement it was decided to design a script to use with the Quantimet 570 Image Analyser. The Q-570 is a fast and accurate image processing and analysis system. Semi-automated, it relieves the investigator of much decision making regarding the discard of irrelevant detail within the image. The manual techniques described above all inherently involve some degree of human error due to the decision making requirement of the investigator. The Q-570 system is therefore not only faster, but more objective and reproducible. It was tested by analysing several images with a manual technique and comparing the results.

3.2 Materials and Methods

3.2.1 Culture preparation

White male Sprague-Dawley rats weighing between 240-250g were anaesthetised using 60mg/ml sodium pentobarbitone given by intraperitoneal injection. They were killed by removal of the heart (performed by a postdoctoral researcher). The optimum handling procedures and techniques for the preparation of the tracheal organ culture were ascertained from previous research in this area (O'Donnell et al., 1973; Mossman and Craighead 1975; Lane and Miller 1976; Pavelka 1976; Chang et al., 1985; Kaartinen et al., 1993). Sterile scissors and forceps were used to remove the skin from the chest area. The underlying tissue was then cleaned using cotton wool soaked in 70% ethanol before another set of sterile scissors and forceps were used to expose the trachea. The trachea was removed from just below the larynx to just prior to the first division of the bronchi. and placed into a bottle of sterile saline. It was then washed in three changes of saline. In each change of saline the bottle was gently inverted for one minute to remove blood and mucus (Toskala, 1995). This ensures that the cilia are not obscured by a covering

of mucus. Extraneous tissue was trimmed from the external surface using sterile forceps and scalpel and approximately 2mm rings cut and halved. The rings at each end of the trachea were discarded due to the likely presence of mechanical damage during the excision of the trachea from the rat with forceps and scissors. Two rings from each trachea were fixed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer for electron microscopy processing before culturing.

The remaining half rings were transferred to 35mm diameter Corning six-well plastic dishes. For each trachea there were six dishes each containing the two halves of a ring. Hanks salts buffered medium 199 (M199, Sigma, Poole, UK) was used in this study. 10% newborn calf serum, penicillin (50 IU/ml), and streptomycin (50 µg/ml) were used to supplement the M199. 1.0 ml of medium was added to each dish. The dishes were then placed in a humid atmosphere (a clear plastic box containing a dampened tissue) in a 37°C incubator containing air. Medium was changed every 24 hours.

The half-rings were examined daily using an inverted light microscope in order to view the beating cilia. Half-rings of trachea were removed from the culture after 1 day, 3 days and after 5 days for observation and analysis by scanning electron microscopy.

3.2.2 Scanning electron microscopy

The tracheal half rings were fixed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.2) for 1½ hours. The cacodylate buffer consisted of 4.28 gram of $Na(CH_3)AsO_2 \cdot 3H_2O$ in 92 cm^3 of distilled water and approximately 8 cm^3 of hydrochloric acid to make the solution up to pH 7.2. The specimens were rinsed in distilled water for 5 minutes and dehydrated in a graded series of ethanol (10-15 minutes in each of 10, 20,

30, 50, 70, 90 and 100% solutions). The specimens were then dried with CO_2 in a critical point drier (Tousimis Samdri-780). Silver paint was used to mount the specimens onto aluminium stubs after which they were coated with gold for 2½ minutes in a sputter coater (Emitech K550) with current of 20mA (nominal thickness 10 nm). The specimens were examined using a Jeol JSM-5200 scanning electron microscope operated at 20 kV.

3.2.3 Image analysis

Each specimen was centred in the screen and nine contiguous images (3 x 3) at a magnification of x2000 were collected by the digital image recording system SemAfore (Jeol). Taking images from the centre of the specimen excluded the consideration of any possible mechanical damage. Each individual image covered an area approximately 645 µm x 414 µm.

Automated image analysis system: Quantimet-570 (Q-570)

The Q-570 is a computerised image processing and analysis system. It is programmed using QBASIC, an interpreter for the programming language BASIC. It runs in conjunction with QUIC under the DOS operating system. Customised scripts can be written, which automate image processing and analysis.

The digital images were transferred into a format suitable for analysis by the Q-570 image analyser (Leica) as follows:

The original images were 1952 x 1500 pixels. The images were opened in a software program called PaintShop Pro and resized using the 'resize image' function. They were resized proportionally to 640 x 480 pixels before saving as a TIFF file. The extension .tiff had to be amended to .tig to enable the Q- 570 to recognise the files (this was simply a

'name change' and did not alter the images in any way). The images were stored on zip disks. A customised macro script was written specifically for the purpose of detecting and quantifying the percentage of each image covered by cilia. Briefly, the images underwent a series of processing stages including extreme sharpening in order to enhance the edges of the objects within. This enabled any irrelevant detail (e.g. microvilli) to be removed. In this way the Q-570 differentiated between ciliated and non-ciliated areas. After 'thresholding', which resulted in a binary image, the quantitation of the ciliated area could take place. The resulting data was then transferred to an Excel datasheet for statistical analysis.

The customised script is detailed below:

1. 10 mframe 0 0 512 478
2. 11 iframe 6 3 502 410
3. 20 mframe 0 0 512 478
4. 21 iframe 6 3 502 410

These instructions define the position and size of the image and measure frame.

5. 30 qmenu 'calibrate'

Defines the relationship between the pixels and user's measurement units (μm in this case).

6. 40 qmenu 'acquire'

Acquisition of stored image (from zip disk) to Q-570.

7. 50 btoph 0 1 256 6 : sub8 0 1 5
8. 51 wtoph 0 1 256 6 : add8 5 1 1

9. 70 wtpoh 1 8 256 6

These instructions serve to sharpen the image making the edges of objects within appear sharper and clearer. The btoph or black tophat transform closes[a] the image by the specified amount (structuring element[b] and size) and the original image subtracted to reveal 'black detail'. The wtoph or white tophat transform opens[c] the image by the specified amount (structuring element and size) and subtracted from the original image to reveal 'white detail'.

[a] Close – A sequence of dilate[d] followed by erode[e] – cleans the image by removing white detail.

[b] Structuring element – fundamental component by which morphological operations are performed – from a group of eight points surrounding a central point it is possible to make the structuring element take any shape. It is positioned on each pixel of the image in turn and a new value calculated for the central point which is then placed in another image.

[c] Open – A sequence of erode followed by dilate – cleans image by removing black detail.

[d] Dilate – Pixels replaced by the local maximum in the neighbourhood defined.

[e] Erode – Pixels are replaced by the local minimum in the neighbourhood defined.

10. 75 greylinopen 8 9 3

11. 76 mgradient 9 10 256 2

Shape detection. These instructions further distinguish between ciliated and non-ciliated areas within the image. A combination of linear openings is performed, removing white detail smaller than the given diameter, preserving long thin features (cilia) while removing spots (microvilli).

12. 80 greydetect 10 34 138 1 1 0

13. 90 qmenu 'detect'

The process of 'thresholding' extracts the binary image from the original grey level image. It was pre-set to detect grey levels from 34 to 138 but could be adjusted if required.

14. 100 binclose 1 2 0 1
15. 110 binerode 2 3 0 12
16. 120 build 2 3
17. 130 binclose 3 4 0 2
18. 140 fillholes 4 5
19. 150 binx 4 5 5 3 0 0
20. 160 binerode 5 6 0 7
21. 170 build 5 6
22. 180 binx 5 6 7 1 0 1
23. 190 binx 4 7 8 2 0 0
24. 200 binsegment 8 9 256 1 6
25. 210 binerode 9 10 0 14
26. 220 build 9 10

Further image processing includes closing, eroding and building of the image in order to further distinguish between ciliated and non-ciliated areas. Ensures non-ciliated areas within larger ciliated areas are not included (finds holes).

27. 224 pausetext 2 'INPUT & OUTPUT AT 10 CUT, DELETE & COVER'
28. 225 qmenu 'bin edit'
29. 226 pausetext 2 ''
30. 230 qmenu 'measure_field'

The pause in the programme allows the user to visualise result of image processing.

31. 240 bininvert 10 11

32. 245 binmove 11 1

33. 250 setftrpar "1, 15, 9, 8, 28, 29, 32, 2, 3"

34. 260 ftrgrey 9 : measfeat 11 1 4 300000 : clraccept

35. 270 acceptxfer 11 12

The final 'processed' image is saved and parameters of feature measurement selected. In this case the measurement of interest is 'proportion' (of ciliated area).

36. 280 qmenu 'feature_results'

37. 290 quic

38. 300 end

Results are displayed, after which the program can be run again.

Alternative image analysis: Image Tool

For the purpose of comparison, the images were also analysed using another computerised program. Image Tool, a simple proportion of area tool, is a freely available program for the PC that enables basic image analysis. The images were opened in the same format as for the Q-570 (i.e. resized and saved as TIFF files). The area of the entire image was then calculated by electronically drawing a box around the periphery. Regions of interest (ROIs) could then also be drawn around the ciliated areas. The number of pixels in each ROI was displayed by Image Tool. Quantitation of the percentage area covered by cilia could then be calculated and the results copied and pasted into an Excel datasheet.

3.2.4 Statistical analysis

Half-rings from three different animals were chosen from the preliminary work for image analysis. From each tracheal half-ring, nine contiguous images were obtained. For each

half-ring therefore, the percentage surface area covered by cilia was an average of nine images.

In order to determine whether there were significant differences in the quantification of percentage cilia coverage between the two image analysis techniques the following statistical tests were applied: Bland-Altman analysis, paired t-tests and ANOVA. All statistical analyses were carried out using SPSS.

3.3 Results

The organ culture described here was well maintained for a period of up to five days as illustrated in Plates 1 - 4. The surface topography of the trachea was similar to that described previously (Alexander et al., 1975; Popp and Martin 1984; Souma 1987).

Due to the different topography observed toward the end of the culture period, images were analysed at the beginning and at the end of the culture period (day 5) using both the Q-570 and Image Tool image analysis techniques. The results of these analyses are shown in Table 3.1.

	Percentage surface area covered by cilia (Mean ± standard deviation)		
	Q-570	Image Tool	Difference between methods
Fresh tissue (day 0) (n= 9)	62.8 ± 10.4%	66.6 ± 8.1%	3.81 ± 4.6% $p< 0.01$
Day 1 (n= 9)	56.0 ± 10.8%	57.3 ± 11.3%	1.26 ± 3.4% $p<0.01$
Day 5 (n= 9)	57.0 ± 11.8%	67.0 ± 13.8%	9.9 ± 4.6% $p<0.01$

Table 3.1 Comparison of Q-570 and Image Tool for assessing percentage cilial coverage in rat tracheal half-rings.

The data were normally distributed ($p> 0.05$, Kolmogorov-Smirnov test for normality).

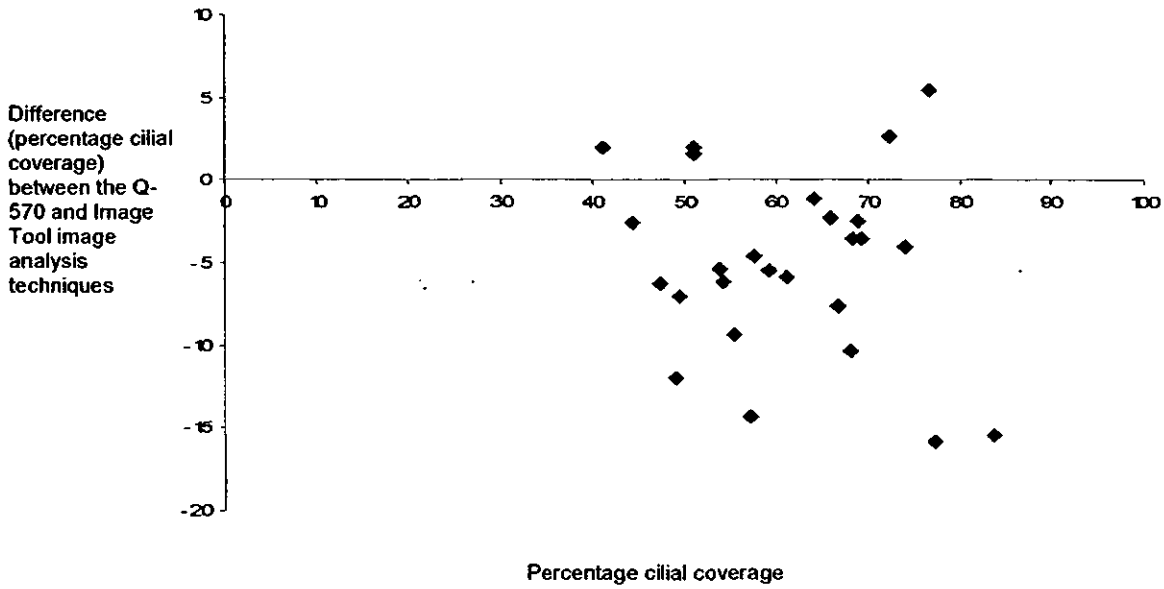


Figure 3.2 Bland-Altman plot comparing two image analysis techniques for assessment of cilial coverage in rat tracheal half-rings.

There was a good correlation between the two methods, which was expected ($r = 0.89$, $p<0.001$). The differences observed between the two methods were statistically significant ($p< 0.01$, paired t-test). The Q-570 appeared to underestimate percentage cilial coverage by an average of 5% (Figure 3.2). There was no correlation between the size of the measurement and the difference between the two methods ($r = 0.17$, $p = 0.42$). There were no significant differences in cilial coverage between days ($p = 0.32$, one-way ANOVA). However the difference between image analysis methods was significantly higher in the images taken from day five than from those taken from fresh tissue or one day into the culture (differences of 9.9, 3.81, and 1.26% respectively ($p< 0.01$, one-way ANOVA).

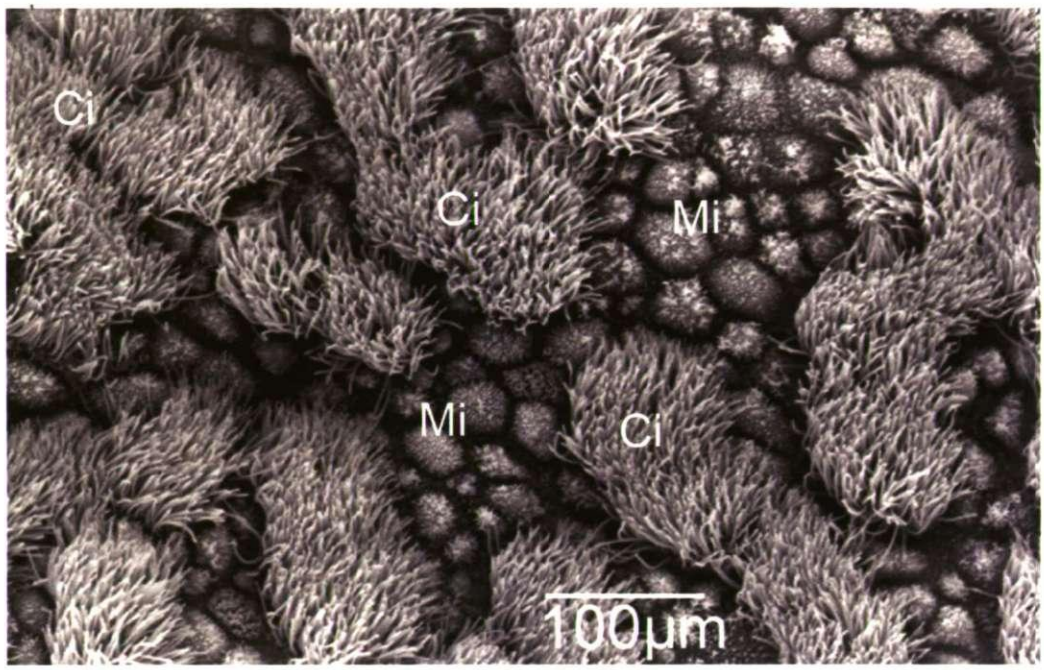
The most frequent difference (mode) was no higher than 5.5% for any of the images.

PLATE 1.

A & B Scanning electron micrographs (original magnification x 2000) showing surface topography of tracheal epithelium in a white male Sprague Dawley rat (fresh tissue). These images are from a representative half ring of fresh rat tracheal tissue. From each half-ring, nine images were obtained and analysed using both the Q-570 and Image Tool techniques.

Interspersed between the clumps of cilia (Ci) can be seen dome shaped cells possessing microvilli (Mi).

A



B

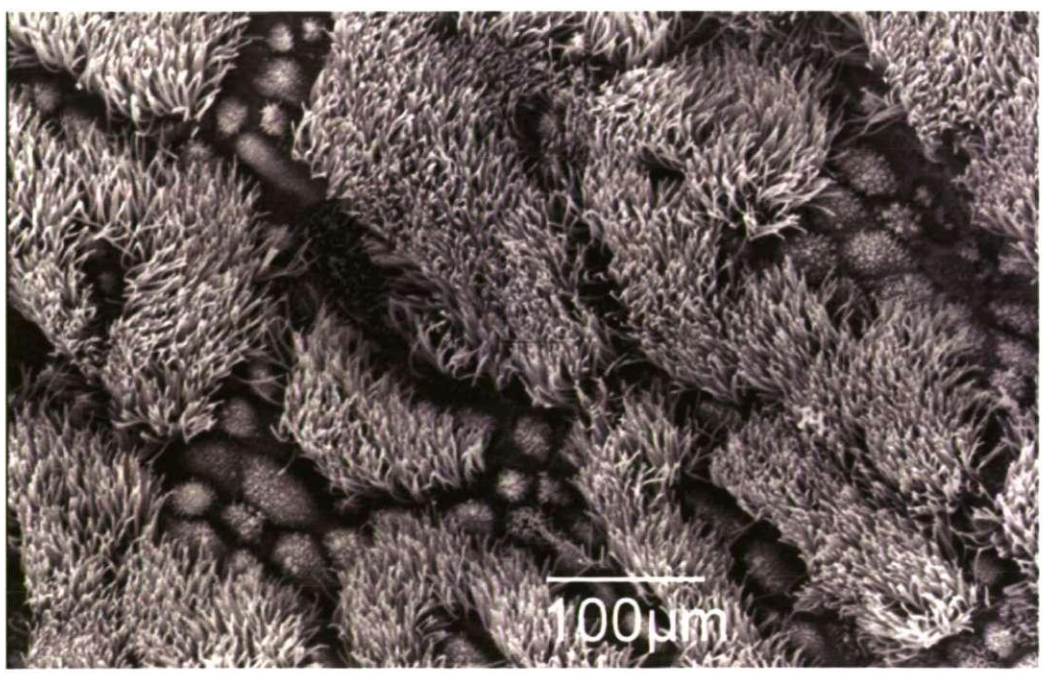


PLATE 2

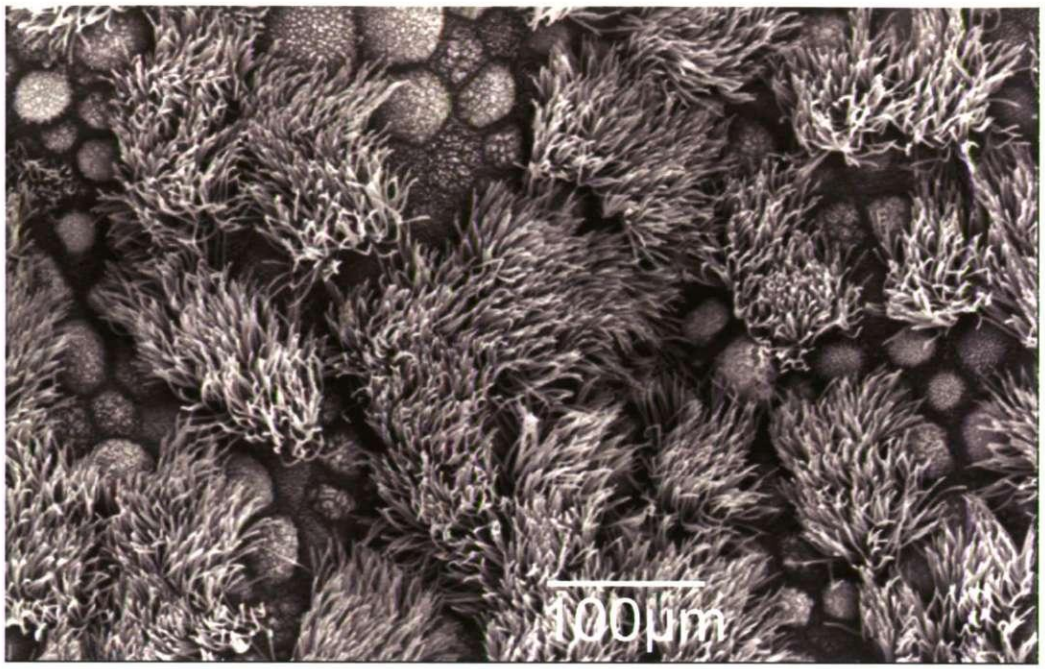
A & B Scanning electron micrographs (original magnification x 2000) showing surface topography of tracheal epithelium in a white male Sprague Dawley rat after 1 day incubation with M199 at 37 °C. These images are from a representative half ring of fresh tracheal tissue at this time point. From each half-ring, nine images were obtained and analysed using both the Q-570 and Image Tool techniques.

These two images have been selected to illustrate the difference in cilial coverage that can occur within one half-ring at a particular time point. There is clearly more coverage in A than in B.

Ci = cilia

Mi = microvilli

A



B

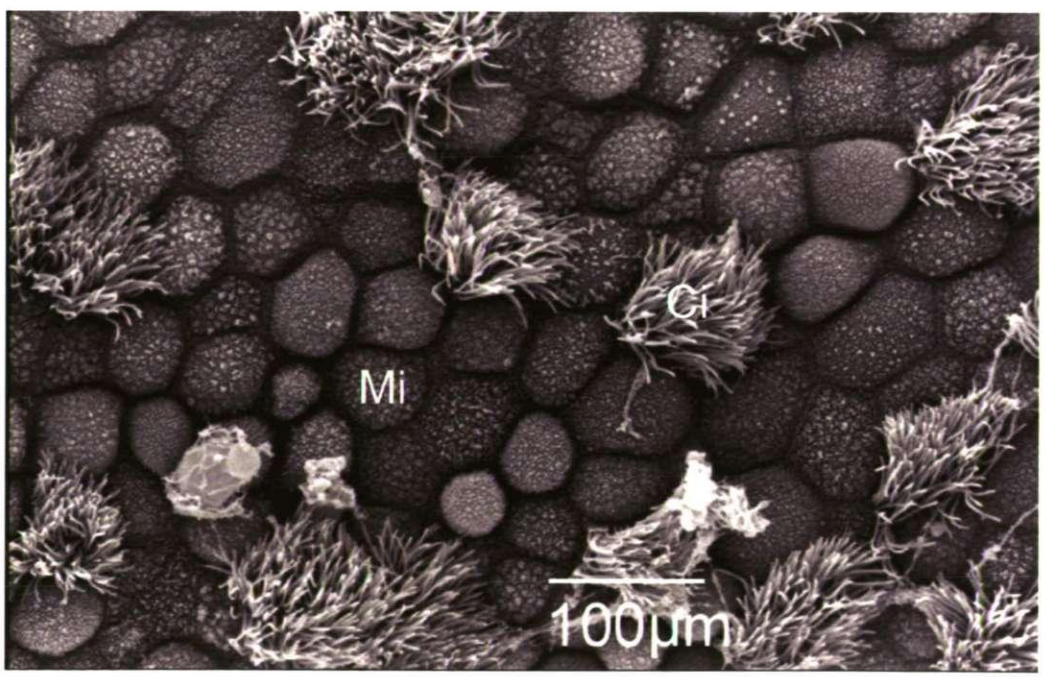


PLATE 3

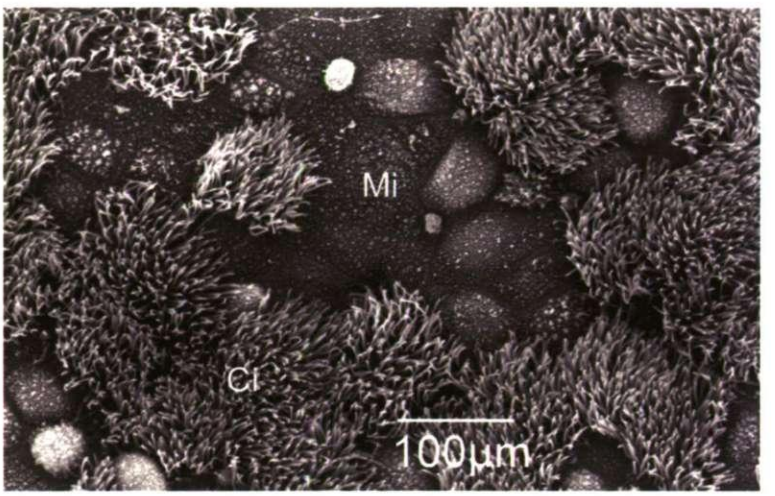
A & B Scanning electron micrographs (original magnification x 2000) showing surface topography of tracheal epithelium in a white male Sprague Dawley rat after 5 days incubation with M199 at 37 °C. These images are from a representative half ring of rat tracheal tissue at this time point. From each half-ring, nine images were obtained and analysed using both the Q-570 and Image Tool techniques.

The surface topography in these images is different to that in the fresh tissue in that the cells in possession of microvilli (or newly forming cilia) are less dome-shaped. These two images were selected to highlight again the difference in cilia coverage that can occur within one half-ring at a particular time point. There is more coverage in A than there is in B.

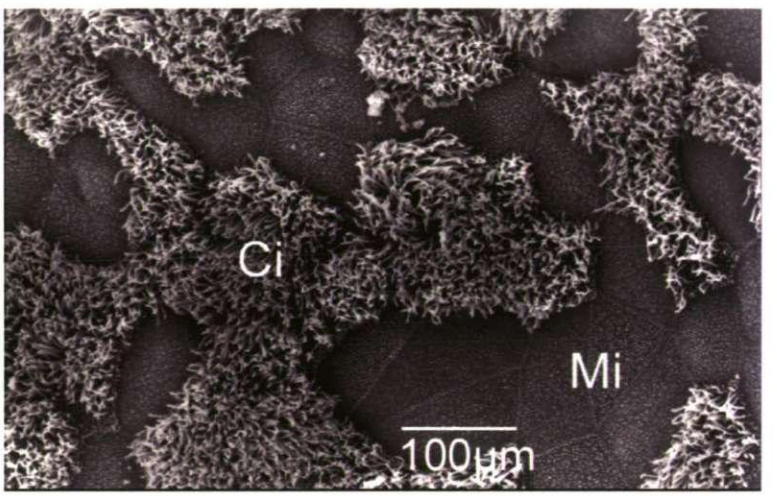
Ci = cilia

Mi = microvilli

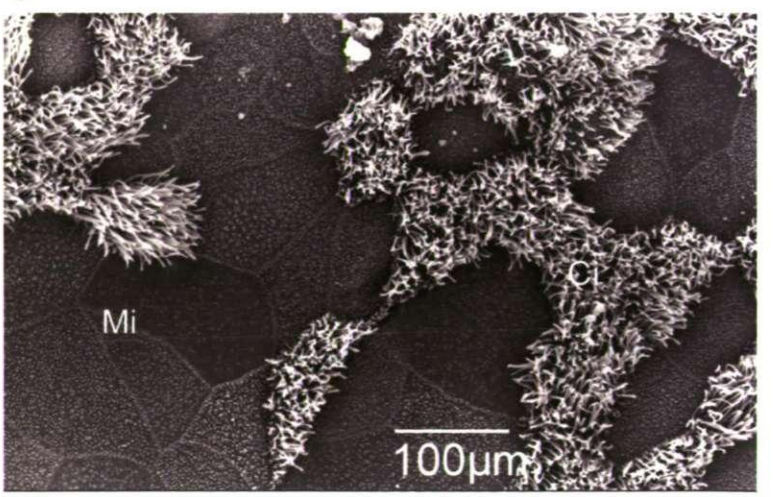
A



B



C



3.4 Discussion

Image Tool was chosen as an alternative means of analysing the images in order to assess the accuracy of the Q-570. Essentially this involved 'tracing' around the objects of interest (cilia), which was very time consuming but has previously been reported to be highly accurate (< 0.5% error) (Glaser and Glaser, 2000). It is reported to be more precise than stereological point counting procedures in area estimation. There was therefore no advantage to performing such a procedure. There was good agreement between the Q-570 and Image Tool methods of image analysis ($r = 0.89$, $p<0.001$). There was a *statistically* significant difference of approximately just 5% between them. However, the alternative method, *Image Tool*, was laborious and could be subjective in nature. The Q-570 was non-subjective and semi automatic. Without any user intervention the repeated analyses would not vary at all whereas the extensive manual input required by Image Tool may lead to variation. One potential drawback of the Q-570 was that there was some difficulty in writing a programme that would simultaneously suit the observed differences in surface topography toward the end of the culture. This is illustrated by the slightly higher difference between the two analysis techniques on the day five images. Overall however, the difference was consistent across the measurement range with most of the differences being approximately only 2%. In order to make certain that accuracy is maintained, particularly at later stages of the culture period, the user can make alterations to the threshold level detection if required. Even so, the Q-570 would still be more objective and certainly quicker than Image Tool. To overcome potential bias of such user alterations it is of course paramount that all subsequent studies are rigorously blinded. The accuracy of the Q-570 was shown to be adequate but the main advantages it had over the Image Tool technique was the minimal decision making required of the investigator and speed of analysis.

Previously described methods of quantitating cilial abundance vary considerably and accuracy is not generally reported. Light microscopy has been utilized, for example, in a study by Weisman and Sade (1979), in an investigation of *in vitro* growth of epithelium, the proportion of the surface area covered by cilia was estimated in histologic sections by assignment of arbitrary units from zero to four. This technique would not be adequate for quantitative studies of cilia survival due to low sensitivity and subjectivity. The accuracy of this technique was not reported. The more recent study by Konrad's group (1995) also utilized a semi-quantitative technique with SEM as outlined in the introduction but also would not be suitable for this research due to similar limitations (Konrad et al., 1995). Their classification of cilial coverage into crude categories would not be sufficient to quantify cilia survival in controlled experiments. Stereological techniques such as those employed by Wilson et al (1984) are limited because they rely heavily on decision making by the investigator, which makes them less reproducible. 'Tracing' techniques for area measurement have been shown to be more accurate and don't take significantly longer to complete than traditional point counting techniques.

Computer assisted image analysis has the benefit of more reproducibility, relying less on investigator judgements which may vary according to tiredness levels and from person to person. Since they can be largely automated, this also makes them quicker. Zahm et al (1990) utilized a computer assisted image analysis technique similar to the one described here but with the added advantage that it was performed on live cells viewed with an inverted light microscope. The images of beating cilia in a cell culture were captured by CCD video camera and digitized. The subtraction and addition of successive images meant that only live ciliated cells were included in the final result (Zahm, et al., 1990). The reproducibility of measurement was reported and was favourable but the technique was not compared to any other. The present technique utilized SEM which is more

suitable for studying the surface topography of an organ culture than LM because it offers a higher resolution and, importantly, a high depth of field. Finally, again in cell culture, Clark et al (1995) stained the ciliated cells of a rat tracheal epithelial (RTE) culture with a monoclonal antibody known as RTE3. CCD images were digitised and analysed with the NIH Image by manually increasing the threshold until areas with no staining began to be highlighted. The resulting image was then subject to binary transformation and the erode command use to remove background pixels. Although quantitative, this technique was not automated and largely subjective. In addition, reliability data were not reported nor was any other technique compared.

In contrast, the present study developed a novel image analysis technique for the assessment of cilial abundance in a rat tracheal organ culture system and quantitatively compared it to a technique known to be accurate to confirm its accuracy and reliability. The results obtained from the Q-570 compared well with those from Image Tool. Despite statistically significant differences between the two methods, most of the differences were just 2%, which is unlikely to be of great clinical consequence. It was a relatively quick, semi-automatic technique which required little user intervention, ensuring minimal subjectivity.

CHAPTER FOUR

ANAESTHESIA

- **Effect of midazolam and propofol on cilia survival *in vitro*.**

- **Effect of halothane upon cilia beat frequency, cilia beat amplitude and synchrony *in vitro* (three – month research studentship in Finland).**

4 Abstract

Certain intensive care anaesthetic agents significantly decrease cilia beat frequency (CBF). Midazolam and propofol have no significant effect on human cilia beat frequency *in vitro*. Loss of cilia has also been associated with impaired mucus transport in intubated patients and commonly used methods of measuring CBF are unable to identify changes in cilia beat amplitude or synchrony. The aims were as follows: to investigate the effect of midazolam and propofol on cilia survival, and to investigate the effect of halothane on CBF, amplitude and synchrony using high speed video. Rat tracheal half-rings in culture were exposed to 150μM midazolam over a period of 5 days. In another experiment, cultures were exposed to 100μM of propofol. Scanning electron micrographs from the half-rings were analysed before and after 1, 3, and 5 days using a customised image analysis script (Quantimet-570) to quantify cilia abundance. *Midazolam*: there was a significant difference between treatments ($p=0.02$), there were no effects due to day or animal ($p>0.05$), 3 factor ANOVA for day, animal and treatment. The slight increase in cilia coverage associated with midazolam is unexpected and of unknown significance. *Propofol*: there was no significant difference between treatments ($p>0.05$). Human tissue was harvested from routine operations and used for cell culture for exposure to halothane. High speed digital video was then used to study the effect of halothane on CBF, amplitude and synchrony. There was a significant decrease in CBF and CBA in the samples exposed to halothane (CBF: 5.6 ± 0.9, 3.9 ± 0.8 ($p= 0.002$); CBA: 2.9 ± 0.1, 2.1 ± 0.6 ($p= 0.003$); synchrony: 2.8 ± 0.2, 2.4 ± 0.4 ($p= 0.07$), ANOVA). Any decrease in MTR associated with midazolam or propofol is unlikely to be mediated by loss of cilia. Decreases in CBF induced by halothane appear to be accompanied by decreases in CBA, the significance of which is not known.

4.1 Introduction

Both inhalational and intravenous anaesthetics have been shown to reduce mucus transport rate (MTR) (Forbes and Horrigan 1977; Cavaliere et al., 1983; Konrad et al., 1998). As such, the duration of anaesthesia contributes to the risk of developing a respiratory infection that intensive care patients face. Furthermore, *in vitro* studies have demonstrated a reversible time and dose-dependent decrease in cilia beat frequency (CBF) during exposure to several inhalational anaesthetic agents including halothane, enflurane, and isoflurane (Raphael et al., 1996). Various parameters affect mucociliary clearance rate including CBF, which is thought to be the main determinant. In addition to CBF, the number of cilia is important for efficient mucus transport (Joki et al., 1998).

There is currently less information concerning the effects of intravenous anaesthetic agents on cilia. Midazolam and propofol are intravenous anaesthetic agents, both commonly used in the intensive care unit to aid sedation during mechanical ventilation. Previous research has indicated that there is no effect on cilia beat frequency (CBF) of human tissue during short term exposure (90 minutes) to propofol or midazolam *in vitro* (Hann et al., 1998). Previous work has demonstrated no effect of midazolam on CBF (Hann et al., 1998) but there is conflicting evidence concerning the effect of propofol (Hann et al., 1998; Shirakami et al., 2000). One study showed no effect (Hann et al., 1998), while another showed an increase thought to be due to stimulation of the NO-guanosine monophosphate (cGMP) signal pathway (Shirakami et al., 2000). Shirakami et al (2000) demonstrated a dose dependent increase in CBF in rat tracheal tissue after just 25 minutes exposure. Exposure to 1 μM propofol caused a 2% increase in CBF, and a dose of 100 μM caused an increase of 14%. Hann et al (1998) also studied short term effects of midazolam and propofol on CBF in human nasal turbinate explants *in vitro* but found no deleterious effect

after 90 minutes exposure to supraclinical concentrations of either drug (20 μM midazolam and 70 μM propofol). These apparently conflicting results may be explained by the species difference and distinct types of tissue used. Hann et al (1998) used human nasal turbinate explants whereas Shirakami et al (2000) used rat tracheal tissues after 3 – 5 days of culture. Further studies ensuing from Hann's work suggested that there may be a deleterious effect of these drugs upon cilia survival (Raphael 1996). During the course of these experiments it was observed that a significant number of the explants exposed to the drugs had no cilia present. Since this was not the primary endpoint of the initial experiments, further studies went on to formally investigate the effect of midazolam and propofol specifically upon cilia survival (Raphael 1996). Subsequent findings were reduced cilia survival after exposure to midazolam but not propofol. There was a time and dose-dependent effect of midazolam upon cilia survival. The concentrations of midazolam for 50% chance of cilia survival were 25 μM after 48 hours exposure and 16 μM after 72 hours exposure. Just three explants out of sixteen survived after 48 hours exposure to 100 μM midazolam. The conflicting results of the latter study were thought to be due to the use of tissue in the original study on CBF, which had been stored for several days before being exposed (Raphael 1996). Storage of the tissue was believed to cause it to be more sensitive to the toxic effects of propofol even though this did not appear to make a difference to the CBF measurements. Crucially, Raphael's study was limited by the crude semi-quantitative method used to quantify the cilia and neither experiment proceeded beyond three days.

Measurement of CBF by the commonly employed transmitted light technique is limited as it measures CBF indirectly and cannot analyse other parameters of ciliary function. In addition to CBF, high speed video has the potential to estimate amplitude (CBA), synchrony, and orientation of the ciliary beat. These parameters all combine to affect the

rate of mucus transport. Rautianen et al have previously described the use of a high speed video system in conjunction with cultured human respiratory epithelial (HRE) cells (Rautiainen et al., 1992; 1993) Their research has indicated that this system is advantageous because all aspects of ciliary function can be studied. Furthermore, the same cells can be observed before and after challenge with test media (Rautiainen et al., 1992). Rautiainen et al (1993) have used high speed video to observe and describe the degeneration of repiratory cell ciliary beat in human monolayer cultures. They found that both CBF and CBA decreased continually and linearly from the first day after plating the culture, but that the waveform did not change. The cell density of the cultures was found to be an important factor controlling the speed of the degeneration. For example, the rate of decline in ciliary function was greater in separate or small groups of isolated cells compared with those within larger groups of cells. High speed video has also been used to study ciliary motility of cultured HRE cells during ciliogenesis and the effect of adenosine triphosphate (ATP) (Yoshitsugu et al., 1993; 1994). Ciliogenesis was observed in cultured HRE cells over a period of fourteen days. It was found that even though CBF reached its peak early in the process of ciliogenesis, the short, immature cilia had a rigid-like movement. Amplitude and synchrony did not peak until days eight and twelve respectively. This illustrates an important advantage of the high speed video system in viewing all parameters of ciliary function since a high CBF does not necessarily indicate optimal amplitude or synchrony. More recently, Chilvers and O'Callaghan (2000) compared measurements of CBF made by high speed digital video technology with the photomultiplier and photodiode (transmitted light) techniques. They found that the latter techniques both under-recorded CBF. In addition they were first to show that respiratory cilia beat forwards and backwards within the same plane, without a classical sideways recovery sweep.

The effect of halothane on CBF *in vitro* is now well established. Gyi et al (1994) exposed nasal brushings to varying concentrations of halothane and found not only a significant decrease in CBF but also that the co-efficient of variation of CBF measurements was increased in samples exposed to halothane (Gyi et al., 1994). This work suggested that there may be additional effects on ciliary co-ordination. Further studies elucidated the reversible dose and time dependent nature of the effect of halothane on CBF (Raphael et al., 1996; 1996). However, nothing is currently known about any additional effects on ciliary co-ordination as suggested by Gyi et al (1994).

The aims of the present study were to investigate the effect of long term (five days) exposure to midazolam and propofol on cilia survival *in vitro* and to utilise high-speed digital video to study the effect of halothane on CBF, CBA and synchrony/ co-ordination.

Further information about the study drugs:

Midazolam (Midazolam-hydrochloride) is a water soluble intravenous benzodiazepine. It has sedative, hypnotic, anxiolytic and amnestic properties and is used for sedation.

Propofol (1, 6- diisopropylphenol) is an intravenous hypnotic used for induction and maintenance of anaesthesia and sedation, and reduces laryngeal tone.

Halothane (2-bromo-2-chloro-1, 1, 1- trifluoroethane) is a volatile inhalational anaesthetic used for maintenance of anaesthesia, and occasionally for induction.

4.2 Materials and Methods

Effect of midazolam and propofol on cilia survival *in vitro*.

Culture preparation

White male Sprague-Dawley rats weighing between 240-250g were anaesthetised using 60mg/ml sodium pentobarbitone given by intraperitoneal injection. They were killed by removal of the heart (performed by a postdoctoral researcher). Sterile scissors and forceps were used to remove the skin from the chest area. The underlying tissue was then cleaned using cotton wool soaked in 70% ethanol before another set of sterile scissors and forceps were used to expose the trachea. The trachea was removed from just below the larynx to just prior to the first division of the bronchi.and placed into a bottle of sterile saline. It was then washed in three changes of saline. In each change of saline the bottle was gently inverted for one minute to remove blood and mucus (Toskala, 1995). Extraneous tissue was trimmed from the external surface using sterile forceps and scalpel and approximately 2mm rings cut and halved. The rings at each end of the trachea were discarded due to the likely presence of mechanical damage during the excision of the trachea from the rat with forceps and scissors. Two rings from each trachea were fixed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer for electron microscopy processing before culturing.

The remaining half rings were transferred to 35mm diameter Corning six-well plastic dishes. For each trachea there were six dishes each containing the two halves of a ring. Hanks salts buffered medium 199 (M199, Sigma, Poole, UK) was used in this study. 10% newborn calf serum, penicillin (50 IU/ml), and streptomycin (50 µg/ml) were used to supplement the M199. 1.0 ml of medium was added to each dish. Medium was changed every 24 hours.

Drug exposure

Supraclinical concentrations of each drug were chosen:

Midazolam

A concentration of 150μM midazolam was made by dilution in the media.

Propofol.

A dilution of propofol was made in media containing 1% dimethyl sulphoxide (DMSO) before addition to the media due to its hydrophobic properties. The concentration of propofol was 100μM.

Three wells were used for drug exposure in each experiment. The remaining three acted as controls. The midazolam controls contained only medium and the propofol controls contained medium and 1% DMSO. The investigator was blinded as to which wells received the media containing the drug.

Two half rings were taken out from each of a well containing the drug and a control, and processed for scanning electron microscopy on days 1, 3 and 5.

Scanning electron microscopy

The tracheal half rings were rinsed in three changes of physiological saline to remove mucus and fixed in 2.5% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.2) for 1½ hours at 4°C. They then underwent standard SEM preparation as detailed in Chapter Three. Briefly, specimens were rinsed in distilled water for 5 minutes and dehydrated in a graded series of ethanol prior to critical point drying with CO_2. They were gold coated (nominal thickness 10nm) and examined using a Jeol JSM-5200 scanning electron microscope operated at 20kV.

Image analysis

Each specimen was centred in the screen and nine contiguous images (3 x 3) at a magnification of x2000 were collected using SemAfore, a digital image acquisition system (Jeol, UK Ltd). The images were then analysed on the Quantimet-570 image analyser (Leica) in order to determine the percentage area of the specimen covered by cilia (Chapter 3).

Statistical analysis

Analysis of variance was used to test for statistically significant differences between the exposed and control samples.

The effect of halothane on CBF, amplitude and synchrony (three-month research studentship in Finland).

A three month visit to the department of Otorhinolaryngology at the university hospital of Tampere in Finland was undertaken in order to take advantage of the latest technique of high speed digital video microscopy for studying ciliary beat and function.

Patients

Tissue for monolayer cell culture was obtained, with prior ethical approval and informed consent, from patients with chronic or recurring sinusitis undergoing nasal or sinus surgery.

Preparation of cell culture

To dissociate HRE cells from specimens, the latter were digested with 6 ml 0.75% pronase (Type 14 protease, Sigma, St. Louis, MO) in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient F-12 (DME/ F-12) supplemented with penicillin (50IU/ ml) and streptomycin (50mg/ ml) at 4 °C overnight.

After removing the specimens, the remaining cells were centrifuged. After removing supernatant, 5 ml media was added and mixed before centrifuging again. Supernatant was discarded again and 3 ml media added. The cell suspension was pre-plated in a plastic dish with the DME/ F-12 medium supplemented with 10% Nu-serum (Collaborative Research Inc., Bedford, MA) and antibiotics at 37 °C for two hours to reduce contamination by fibroblasts, which more avidly adhere to the plastic surface than do epithelial cells (cell density approximately 5000/ cm^2).

The epithelial cells, in suspension in 2 ml of the DME/ F-12 medium, (supplemented with

10 ng/ ml cholera toxin (Sigma) 10^{-7} M retinoic acid (Sigma), 10% Nu-serum, (collaborative Research Inc, Bedford, MA) and antibiotics (50IU/ ml penicillin and 50 µg/ ml streptomycin)), were seeded on a collagen gel layer as a growth substrate in culture dishes having a diameter of 35 mm. Cholera toxin and retinoic acid have previously been shown to promote respiratory epithelial proliferation and differentiation *in vitro* (Jeffery et al., 1977; Kaartinen et al., 1993; Clarke et al., 1995). Type I native collagen (2.3 mg/ ml) was prepared from rat tail using a procedure by Montesano and Orci (1985) by the host laboratory. Approximately 1 ml was added to the culture dish containing a coverslip, and then the excess removed so that the bottom of the dish was just covered. The dish containing the collagen was incubated at 37 °C for thirty minutes until gelled. The cells were cultured at 37 °C in an atmosphere of 5% CO_2. The medium was changed with 2 ml of the medium one day after plating and subsequently every other day.

Measurements

Cells were observed with an inverted microscope (Nikon diaphot – TMD, Tokyo) and an oil immersion objective with a magnification of x 100. Prior to observation the coverslip containing the culture was removed from the culture dish and placed into a custom made viewing chamber. A minimum of ten different cells within each culture were recorded with high speed video for analysis of cilia beat frequency (CBF), cilia beat amplitude (CBA), and synchrony. CBF was measured during playback of the recording at slow speed by direct counting of the beats. CBA and synchrony were estimated by classification into distinct groups as follows:

Table 4.1 Classification of CBA and synchrony in HRE cultures

CBA		Synchrony	
0	Cilia barely moving at all, amplitude of beat appears 'tremor-like'	1	None – cilia in close association beating in totally random directions to each other and not in the same phase of beat at the same time.
1	Cilia beating with poor but just recognizable amplitude.	2	Some – but not most of the cilia beating in a common direction and in synchrony with each other.
2	Good – clearly recognizable amplitude.	3	Good – Most of the cilia beating in the same direction and in synchrony with each other.
3	Arc of cilia beat very wide.		

Drug exposure

The monolayer cultures, on glass cover-slips, were supported in a custom made viewing chamber. Exposure to the drug took place inside a sealed vessel of known volume (2750 ml). The appropriate amount of halothane required in order for a concentration equivalent to 3 MAC (2.25%) to be achieved was added to a dish inside the chamber (1 ml halothane = 226.5 ml vapour (Halsey, 1996), therefore 0.27 ml required).

Recordings were taken 1 day after plating, before and after the exposure to 3 MAC halothane for four hours. The same ten areas of each culture were examined where possible.

Statistical analysis

Differences in CBF, CBA, and synchrony before and after the experiments were analysed using ANOVA. Relationships between the variables were explored using simple linear regression.

4.3 Results

Effect of midazolam and propofol on cilia survival *in vitro*.

The results are shown in Table 4.1. There was a statistically significant higher percentage surface area covered by cilia in the tracheal half-rings exposed to midazolam compared to the control on day 3 only ($p= 0.02$). There was no effect on cilia survival after exposure to propofol ($p>0.05$).

The control values for percentage cilia coverage were similar to previous observations during the course of developing the culture and image analysis techniques (Chapter Three). No other unusual observations were made during SEM of any of the tracheal half-rings.

Table 4.2 Mean percentage cilia coverage in rat tracheal half-rings exposed to 150 μM midazolam in M199 and 100 μM propofol in M199 containing 1% DMSO.

	Percentage surface area of rat tracheal half-rings covered with cilia (%) Mean ± SD (95% confidence interval)			
Day	Control n= 10	Midazolam n= 10	Control n= 6	Propofol n = 6
0	60.9 ± 10.9 (52.7 – 69.1)	60.9 ± 10.9 (52.7 – 69.1)	51.9 ± 11.2 (40.1 – 63.7)	51.9 ± 11.2 (40.1 – 63.7)
1	65.3 ± 8.8 (58.6 – 72.0)	66.1 ± 9.4 (59.1 – 73.2)	63.8 ± 22.7 (40.0 – 87.6)	61.7 ± 22.0 (38.6 – 84.8)
3*	61.3 ± 14.6 (50.3 – 72.4)	77.7 ± 9.6 (70.4 – 84.9)	68.8 ± 15.5 (52.3 – 85.0)	71.3 ± 13.4 (56.9 – 85.6)
5	72.9 ± 10.8 (64.8 – 81.1)	75.9 ± 11.1 (67.5 – 84.3)	71.7 ± 22.8 (35.5 – 108.0)	65.5 ± 22.2 (30.1 – 100.1)

*** The only statistically significant difference was on day 3 ($p= 0.02$, ANOVA) in the midazolam experiment.**

The effect of halothane on CBF, amplitude and synchrony (three-month research studentship in Finland).

Seven cultures were successfully utilised for exposure to halothane. The results are shown below:

Table 4.3 CBF, CBA, and synchrony in HRE cultures exposed to 3 MAC halothane and control - mean ± standard deviation (95% confidence interval).

	Control before	n= 6 after 4 hrs	p	Halothane before	n= 7 after 4 hrs	p
CBF (Hz)	5.8 ± 0.8 (5.0 – 6.7)	5.2 ± 0.8 (4.4 – 6.1)	0.22	5.6 ± 0.9 (4.8 – 6.5)	3.9 ± 0.8 (3.2 – 4.6)	0.002
CBA	2.9 ± 0.1 (2.8 – 3.0)	2.7 ± 0.3 (2.4 – 3.0)	0.13	2.9 ± 0.1 (2.8 – 3.0)	2.1 ± 0.6 (1.6 – 2.7)	0.003
Synchrony	2.7 ± 0.2 (2.5 – 2.9)	2.7 ± 0.2 (2.5 – 2.9)	0.88	2.8 ± 0.2 (2.6 – 3.0)	2.4 ± 0.4 (2.0 – 2.8)	0.067

P-values indicate significance of difference in the parameters before and after 4 hours incubation (ANOVA).

CBF, CBA, and synchrony remained stable over a period of four hours in the control group. There was a significant decrease in CBF and CBA before and after halothane exposure as well as a slight decrease in synchrony, which was not statistically significant.

In the control group, there was a positive correlation between CBA and CBF (Figure 4.1). In the group exposed to halothane there were correlations between synchrony and CBF (Figure 4.3) and between CBA and synchrony (Figure 4.4) in addition to between CBA and CBF (Figure 4.2).

Figure 4.1 Scatter plot of CBF and amplitude of cilia beat in human respiratory epithelial cell cultures (control).

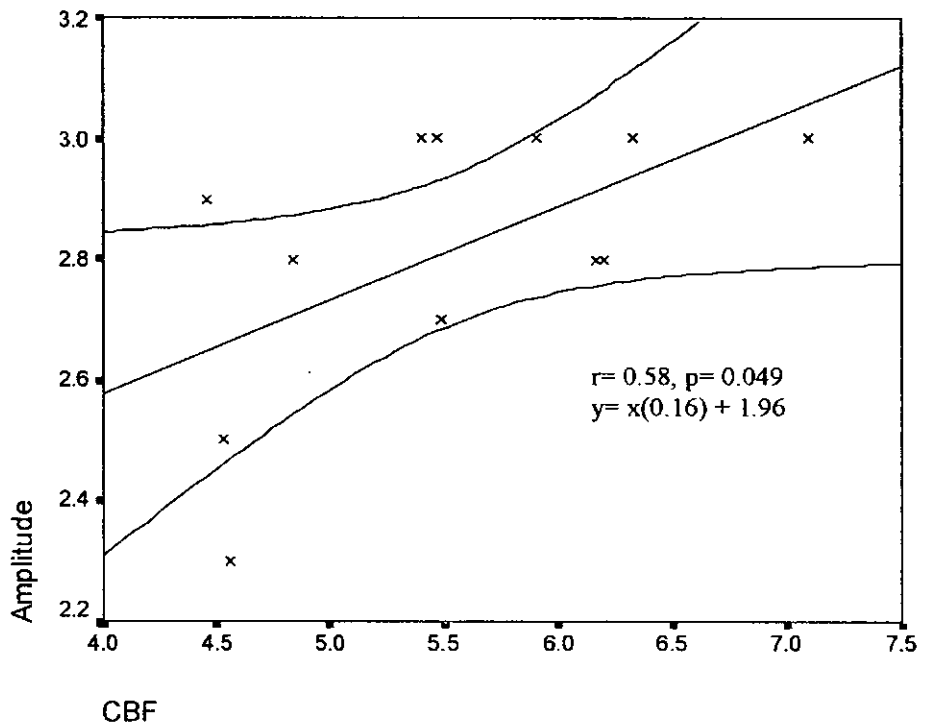


Figure 4.2 Scatter plot of CBF and amplitude of cilia beat in human respiratory epithelial cell cultures exposed to halothane.

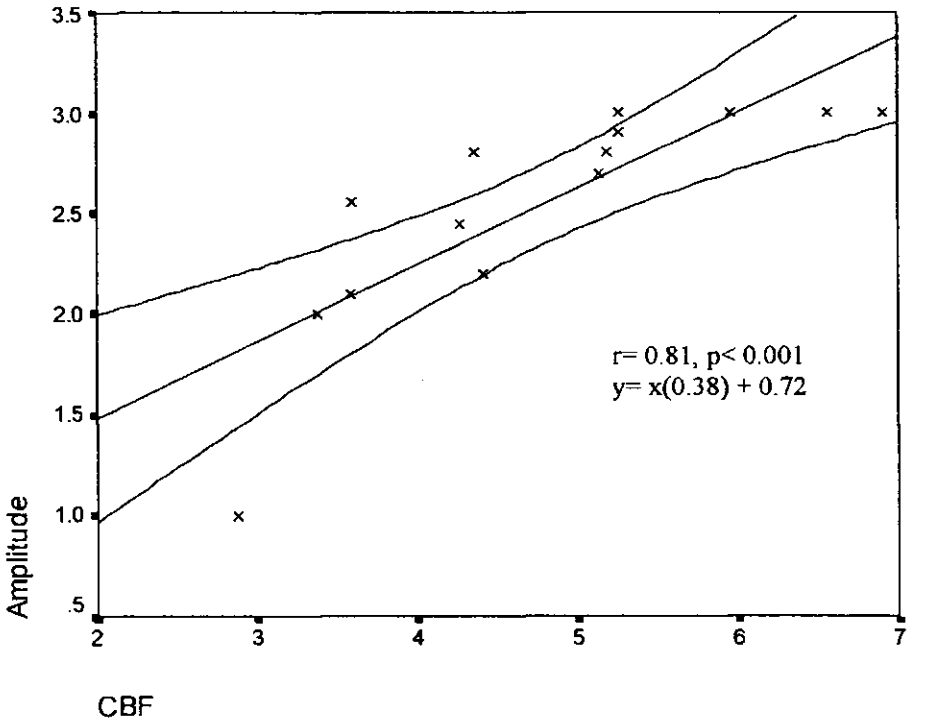


Figure 4.3 Scatter plot of CBF and synchrony of cilia beat in human respiratory epithelial cell cultures exposed to halothane.

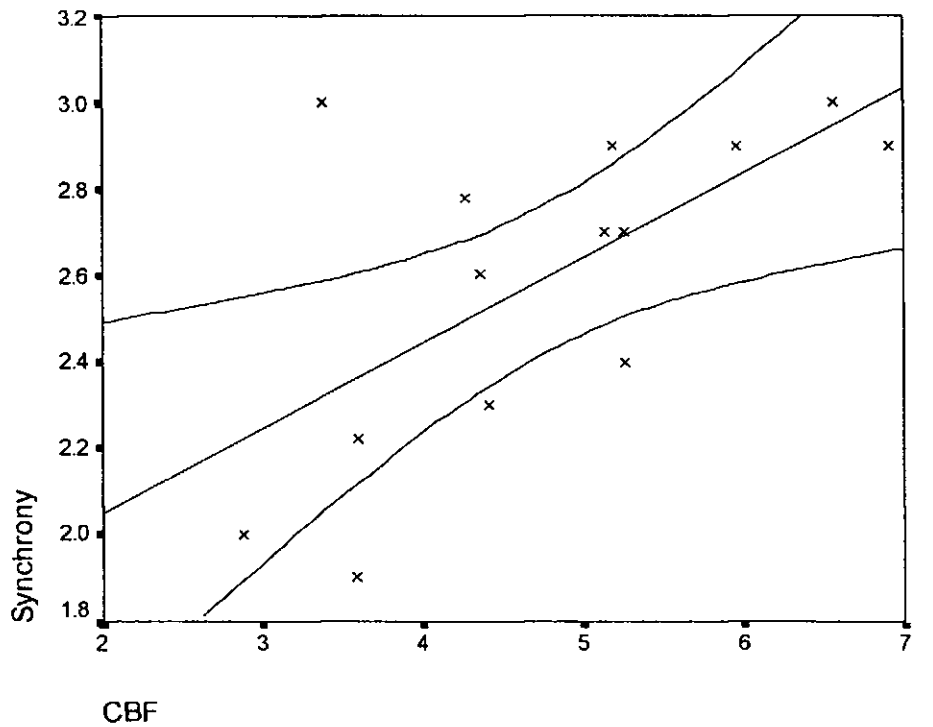
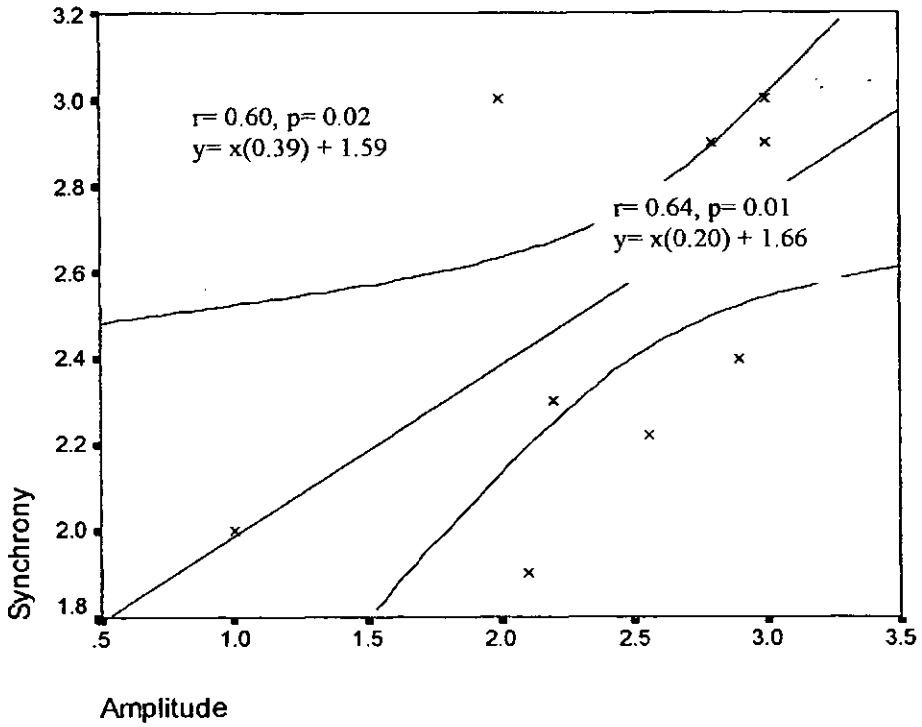


Figure 4.4 Scatter plot of amplitude and synchrony of cilia beat in human respiratory epithelial cell cultures exposed to halothane.



4.4 Discussion

There may be several reasons why the preliminary findings of Raphael et al (1996) were not confirmed in the present study. Raphael et al (1996) used human nasal turbinate explants, whereas the present study utilised a rat tracheal organ culture system. Perhaps most importantly, unlike the previous study, the present work utilised a robust quantitative technique. The previous study used only light microscopy and a crude scoring system. The explants were simply examined for the presence or absence of cilia and each explant was classified as either having or not having any visible cilia. If no cilia were clearly visible, the explant was defined as not having survived. The use of SEM enabled observation and analysis of the mucosa in much more detail such that any additional morphological damage to the surface would have been detected, and the amount of cilia could be reliably quantified.

It is possible that the drugs were unstable in the media but this was considered in previous work, in which fluorimetric analyses showed that there was minimal loss from the containers (Raphael 1996). This possibility was further minimised by changing the medium along with freshly diluted study agent everyday.

Propofol was dissolved in DMSO due to its hydrophobic properties, however in clinical practice this drug is administered in an aqueous solution of soya bean oil, glycerol and egg phoshatide. This formulation may have a different effect on the cilia than that used in this experiment. Further work could investigate this possibility.

The present study was a novel investigation and there is no other literature on the effects of midazolam and propofol upon cilia survival. It therefore represents significant

advancement of knowledge in this field. In addition, there is little information available on the effects of midazolam and propofol upon mucociliary clearance. (Konrad et al., 1992) measured bronchial mucus transport velocity (BTV) in patients ventilated with midazolam, fentanyl, pancuronium, and nitrous oxide. Radiolabelled microspheres were deposited on the dorsal mucosal surface at the distal end of the right and left main bronchus and their movement towards the trachea was visualised with a scintillation camera. BTV was measured in this way before and after ventilation and no adverse effect of general anaesthesia on BTV was detected. However, this was only a short term study and did not examine the effects of midazolam alone. (Hasani et al., 1992) investigated the effect of temazepam, a related benzodiazepine, on mucociliary clearance in healthy volunteers. They assessed mucociliary clearance by measuring the change in radioactivity in the lungs after inhalation of radio-labelled particles and compared clearance rates between temazepam and placebo administration. Temazepam reduced tracheobronchial clearance by 22% compared with placebo. More work is needed to assess the individual effects of midazolam and propofol on mucociliary clearance.

The implications of the findings presented here are important and further work is also needed to confirm that any decrease in MTR associated with midazolam or propofol is unlikely to be mediated by loss of cilia or decreased CBF.

Halothane was chosen in the work which utilised the high speed video system in Finland initially because its effect on CBF and mucociliary function is already well established in the literature (Forbes 1976; Manawadu et al., 1979; Lee and Park 1980; Gyi et al., 1994; Cervin et al., 1995; Raphael et al., 1996). Comparable results would validate the techniques used as dependable means by which to test similar agents.

The CBF values were overall significantly lower than those described in these types of experiment previously. This is most likely to be due to the fact that measurements were carried out at room temperature and CBF is well-known to be temperature dependent (Clary Meinesz et al., 1992). There was a significant decrease in CBF in cultures exposed to halothane but not statistically significant decrease in synchrony while the controls exposed to air remained stable over the same time period. Previous work has already elucidated a dose and time dependent relationship *in vitro* (Raphael et al., 1996; 1996), which is consistent with findings of decreased MTR *in vivo*. Raphael et al (1996) demonstrated a reduction in CBF of human nasal cilia of 40% after two hours of exposure to 3 MAC halothane. Earlier work by their group had demonstrated a 20% and 58% decrease after two hours exposure to 1.8% and 5.7% halothane respectively. The results of the present study are consistent with these observations. After four hours of exposure to 3 MAC halothane CBF in the monolayer cell culture was decreased by approximately 30%. A concomitant decrease in amplitude and synchrony may help to explain why such an apparently small decrease in CBF leads to a large decrease in mucus transport rate.

It is thought halothane could cause this decrease in CBF via a direct toxic effect on ion channels in the ciliated epithelium (Pizov et al., 1992). Halothane has been shown to decrease ion and water transport in dogs and impaired fluid secretion is associated with decreased MTR (Pizov et al., 1992). Accumulation of Ca^{2+} and ATPase activity have also been shown to be inhibited by halothane in sarcoplasmic reticulum (Malinconico and McCarl 1982). If halothane has the same effect on dynein, an ATPase, which is the motor of ciliary movement, this too could help explain the observation.

In addition to the decrease in CBF, CBA was also significantly decreased in the cultures exposed to halothane. Although statistically significant, the clinical relevance of this small decrease is not known. In the group exposed to halothane there were significant correlations between CBA and CBF, synchrony and CBF, and CBA and synchrony. A significant correlation between CBA and CBF was also found in the control group indicating that decreases in CBA and synchrony accompany decreases in CBF. Other literature supports this observation (Rautiainen et al., 1993) which may help to explain why small decreases in CBF lead to large decreases in MTR (Duchateau et al., 1985).

In summary, this investigation has found for the first time that decreases in CBF caused by exposure to halothane are likely to be associated with decreases in CBA. However, the results should be interpreted with caution due to the measurement conditions and the technique does have an important limitation. CBA and synchrony were assessed semi-quantitatively and possibly subject to observer error. The subjective nature of this measurement could be alleviated by utilisation of automated image analysis. This would improve both accuracy and robustness of the technique.

The present study was also limited by the fact that only two measurements, before and after exposure, were made. In order to demonstrate the timing of effect of agent on the cilia, it would be beneficial to set up a perfusion chamber similar to that used previously. This, in conjunction with the cell culture and high speed video system, would enable the same cells to be observed during exposure. It would negate the need to remove the specimens from exposure and would allow more frequent measurements to be made.

Together the results of these investigations in conjunction with previous published work

suggest it may be therapeutically advantageous to the ICU patient if midazolam or propofol be used in preference to halothane. If halothane has a similar effect on cilia at clinical concentrations *in vivo* as in the present study, it is likely that mucociliary clearance would undoubtedly suffer with prolonged exposure. In fact, it has already been shown to reduce mucus transport rate *in vivo* (Forbes and Horrigan 1977; Cavaliere et al., 1983). Conversely, if midazolam and propofol have a similar effect on cilia abundance at clinical concentrations *in vivo*, mucociliary clearance is unlikely to be adversely affected. Indeed there is evidence to suggest CBF is not adversely affected by either of these two agents although their effect upon *in vivo* mucociliary clearance, in contrast to halothane, is less clear.

CHAPTER FIVE

ABNORMALITIES AND ORIENTATION OF CILIA

IN NON-SYMPTOMATIC SMOKERS AND NON-SMOKERS.

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5 Abstract

Smoking may induce the formation of abnormal cilia, which may predispose smokers to nosocomial infections and/ or represent an early stage in the pathogenesis of diseases commonly associated with smoking. The ultrastructure of tracheal cilia was studied in brushings taken from asymptomatic smokers and non-smokers undergoing routine day case surgery. The proportions of abnormal cilia in the smokers and non-smokers were 3.21% (median 2.77, range 0.52-8.37) and 3.05% (median 2.89, range 0.76-7.21), respectively. There was no significant difference between the groups ($p= 0.996$, unpaired t-test after logit transformation). None of the patients' cilia could be classified as grossly disorientated and the variation of cilial orientation in the two groups was similar. There was a slight upward trend in the number of pack years smoked and proportion of cilial abnormalities but this was not statistically significant. Although there was no difference between this group of smokers and non-smokers, this finding may suggest that a larger number of asymptomatic smokers with a higher pack years of smoking need to be studied.

5.1 Introduction

Cigarette smoking has long been associated with an increased risk of developing respiratory infections in the post-operative period and in intensive care (Pearce and Jones 1984; Dilworth and White 1992). The adverse effect of tobacco smoke on mucociliary clearance *in vitro* and *in vivo* is also well established (Dalhamn 1970; Wanner 1985). Denudation of the cilia, squamous metaplasia, and mucus hypersecretion are associated with smoking. In the long term, cigarette smoking is associated with mucus transport rates of around 30% that of non-smokers, though there is conflicting evidence as to the effect of short term exposure (Auerbach et al. 1979; Wanner 1985). However, although morphologic changes of the respiratory epithelium have been described there is little work on the effects of smoking on the ultrastructure of cilia. Studies comparing ultrastructural abnormalities in smokers and non-smokers have yielded conflicting results though ultrastructural abnormalities of cilia have been reported in patients with bronchitis for which smoking is the main risk factor. Attempts have been made previously to establish whether or not tobacco smoke induces an increased amount of cilial abnormalities and they have been described in conditions for which smoking is a significant risk factor. For example, Lungarella et al (1983) studied the ultrastructure of bronchial cilia in patients with bronchitis and found that the amount of ciliary abnormalities was significantly higher in patients with chronic bronchitis compared with non-smoking controls but found no difference between bronchitic smokers and non-smokers. The percentage abnormal cilia ranged from 8-28% in the symptomatic patients compared to 0-6% in the non-smoking controls. The lack of a difference in percentage abnormal cilia between the smokers and non-smokers in the symptomatic patients in this study may be due to the very fact that they were ill. All the asymptomatic patients were non-smokers. A study by Trevisani et al

(1992) found a significantly higher percentage of abnormal cilia in chronic bronchitic smokers (7%) compared with asymptomatic smokers (4%). It was noted in this study that the control patients smoked significantly less (53.04 pack years in the bronchitics, 22.62 in the asymptomatic smokers), which presumably accounts for their lack of symptoms. It is thought that the abnormal cilia found in asymtomatic smokers may be an early manifestation of disease. There is however little evidence of a difference between smokers and non-smokers prior to the onset of disease. Rossman et al (1983) studied cilial abnormalities in nasal brushings from a range of patients including asymptomatic smokers and non-smokers between which no difference was observed. In contrast, it was suggested by Verra et al (1995), that tobacco smoke does induce cilial abnormalities and further, that such abnormalities were not reversible following cessation of smoking (unlike the reversible nature of abnormalities acquired during microbial infection). They found the percentage of cilial abnormalities to be significantly higher in smokers and ex-smokers (16.5% and 17.5%) than in non-smokers (5.2%) with chronic sputum production and healthy controls (0.7%).

This is a blinded study of 50 healthy patients carried out to determine whether there is an increased incidence of ciliary abnormalities in asymptomatic smokers than non-smokers. Asymptomatic smokers were defined by there not being any clinically significant chronic respiratory disorder.

5.2 Method

5.2.1 Patients

Day surgery patients aged between 18 and 62 attending Plymouth's Derriford Hospital were recruited into this study (n=50; 13 male, 37 female) and asked to complete a questionnaire (page 126) related to their smoking habits. In addition, the smokers were asked for how long they had smoked. Pack years smoking was then calculated from the number of 20 cigarette packs smoked by a patient per day and the number of years the patient has smoked, with one pack per day, for one year, equating to one pack year.

5.2.2 Specimen collection

A sheathed cytology brush was wet with isotonic saline before being used to obtain a sample of ciliated epithelium from the trachea of patients immediately following induction of anaesthesia (propofol, fentanyl, isoflurane, N_20). The patient group consisted of 50 healthy smokers (7 male, 16 female) and non-smokers (6 male, 21 female). The adherent tissue was dislodged from the brush by brisk agitation into a bottle containing 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.2).

5.2.3 Ultrastructure of cilia

Specimen preparation

Specimens were fixed for 2-3 hours. Following centrifugation supernatant glutaraldehyde was removed and the tissue rinsed in three changes of cacodylate buffer. Following post-fixation with osmium tetroxide for 1 hour, the tissue was rinsed in water for 10 minutes after which it was routinely processed for transmission electron microscopy: dehydrated in a graded series of ethanol and embedded in epoxy resin.

Semi-thin section sections stained with methylene blue were examined by light microscopy in order to locate areas containing transverse cross-sections. Ultra-thin sections were taken from five different areas within each of the tissue blocks at least 10 μm apart to ensure no area was assessed twice. The sections were collected onto copper mesh grids and stained with 2% uranyl acetate for 10 minutes and 1% lead citrate for 15 minutes before examination in a JEOL 1200 transmission electron microscope operated at 80kV.

Analysis

Two areas, each containing at least 50 cilia cut in transverse cross-section, were assessed from within each section. The abnormalities were counted and classified into the following groups: peripheral anomalies (missing/ extra peripheral tubules), central anomalies (missing/ extra central tubules), miss-arrangements of axonemal structure (with no missing/ extra tubules), compounds (more than one 9 + 2 arrangement within a membrane), combined (combination of any of the above), and miscellaneous. The number of abnormalities were expressed as a percentage of the total number of cilia counted in that patient. Orientation was determined in an arbitrary sub-set of 30 patients by obtaining micrographs at a magnification of x30, 000 at which at least 10 clear transverse cross-sectioned cilia could be identified. The negatives were scanned into a computer (Apple Mac) and lines electronically drawn through the central pair of microtubules (the direction of beat is perpendicular to this line). Image analysing software (NIH Image) was then used to calculate the angles of the lines relative to a reference line (the horizontal). The degree of orientation was determined by calculating the standard deviation of these measurements. Plates 6 and 7 illustrate the method of orientation measurement.

Laboratory researchers were blind to patient details until analysis was complete.

PATIENT SMOKING QUESTIONNAIRE

Do you smoke?..... Yes/ No

If yes, please detail.....Type of cigarette/ cigar smoked.....

Number smoked per day.....

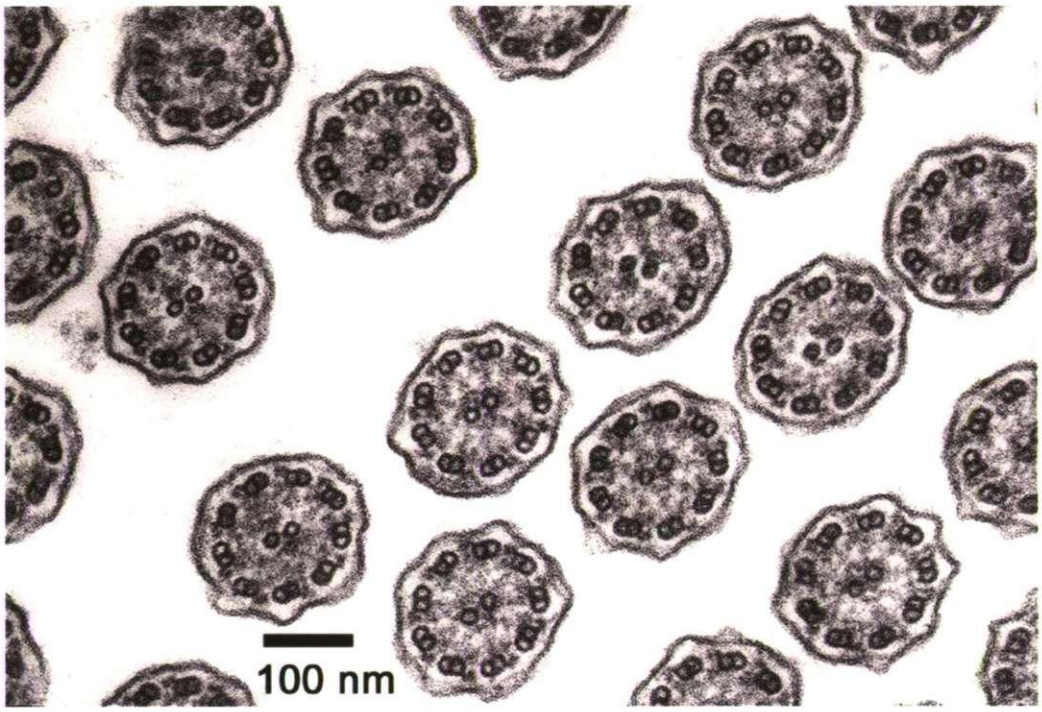
If no, does anyone in your household smoke?.....

Plate 4

A Transmission electron micrograph showing transverse cross-sections of normal cilia.

B Transmission electron micrograph showing lines electronically drawn through the central pair of microtubules in transverse cross-sections of cilia.

A



B

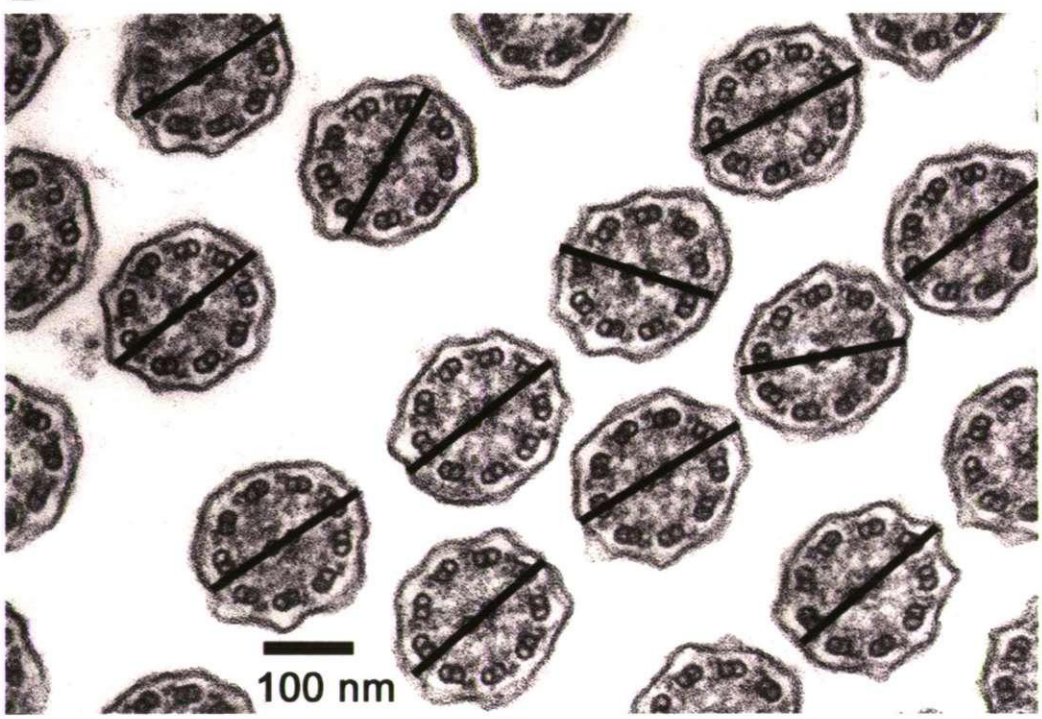
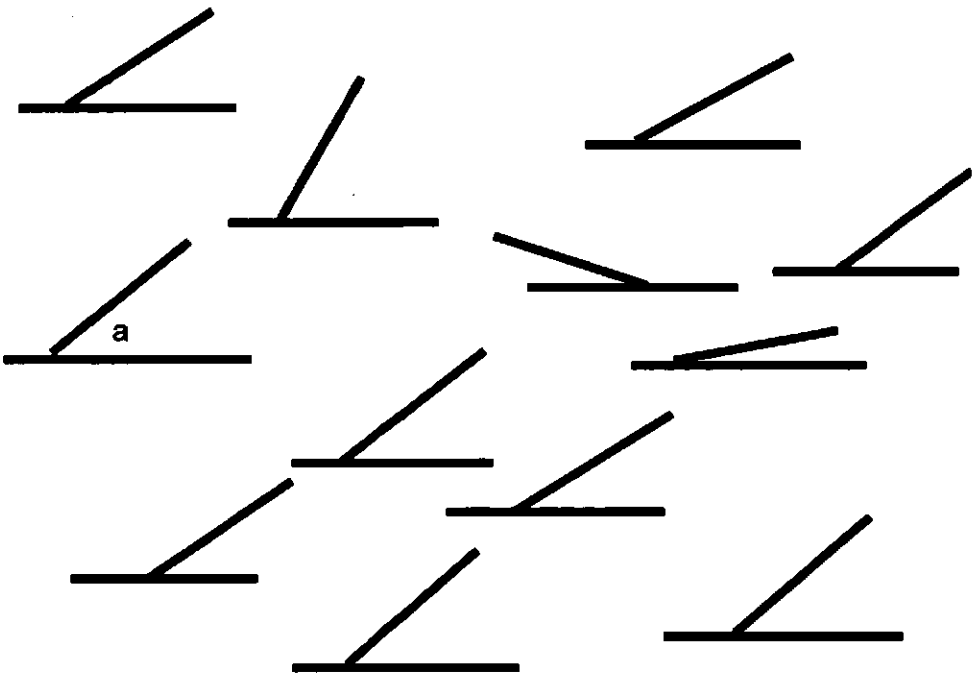


Plate 5

A The angles (a) between the electronically drawn lines and the horizontal can then be calculated. The standard deviation of these angles is a measure of orientation.

A



5.3 Results

The mean age and smoking habits of the patients are shown in Table 5.1. The study population consisted of 23 smokers and 27 non-smokers. Of the 27 non-smokers, just 4 could be classified as passive smokers (with at least one member of their household being a smoker) and only 5 as ex-smokers. With such small numbers in the latter two groups it was not considered appropriate to analyse them separately.

Table 5.1 Characteristics of the smokers and non-smokers

	Gender		Age, years		Smoking habit, pack years	
	M	F	Mean	SD	Mean	SD
Smokers n= 23	7	16	29.6	7.2	6.8	4.9
Non-smokers n= 27	6	21	34.7	10.3	0	0

5.3.1 Ultrastructural abnormalities

The mean percentage of abnormal cilia was 3.21% (median: 2.77, range: 7.85) in the smokers and 3.05% (median: 2.89, range: 6.45) in the non-smokers. The data were not normally distributed and were therefore subject to a logit transformation before statistical analysis. There was no significant difference in incidence of abnormal cilia between the two groups ($p = 0.996$, t-test). Table 5.2 details the different types of abnormalities encountered.

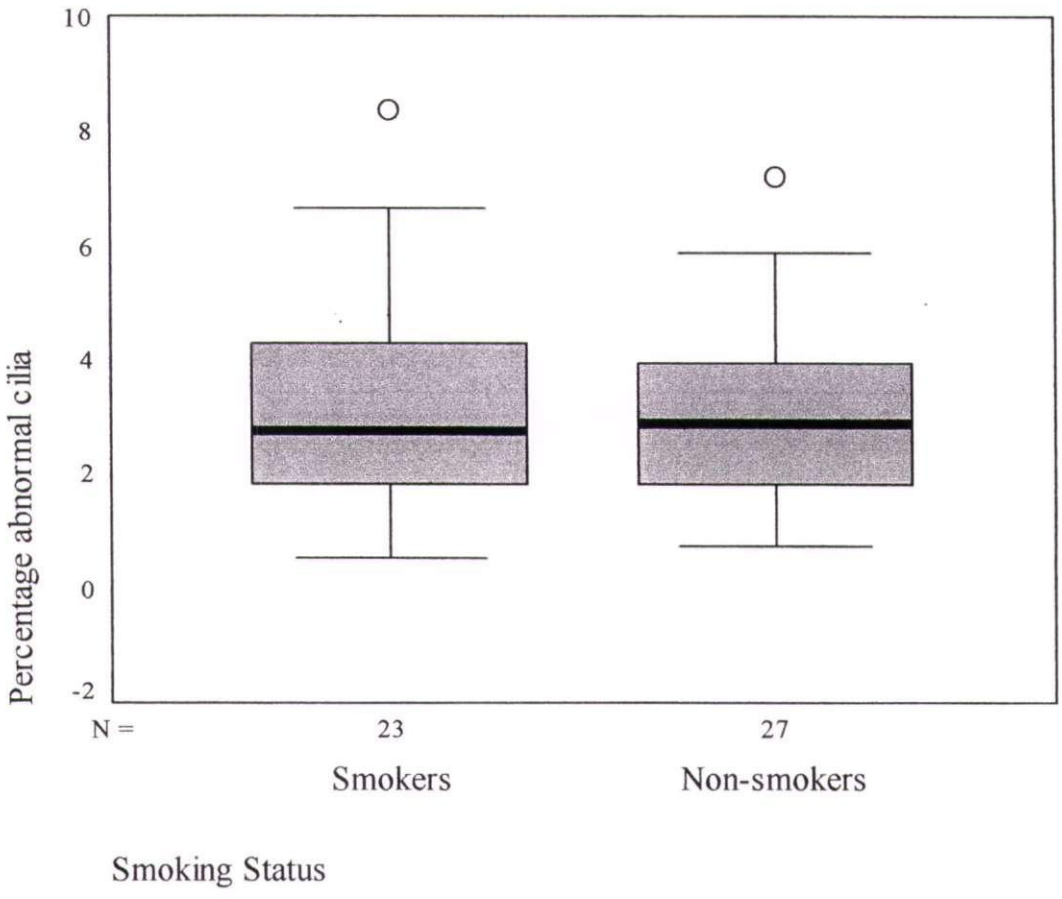


Figure 5.1 Box-plot showing incidence of abnormal cilia (percentage) in asymptomatic smokers and non-smokers. There was no significant difference in incidence of abnormal cilia between the two groups (p= 0.996, t-test).

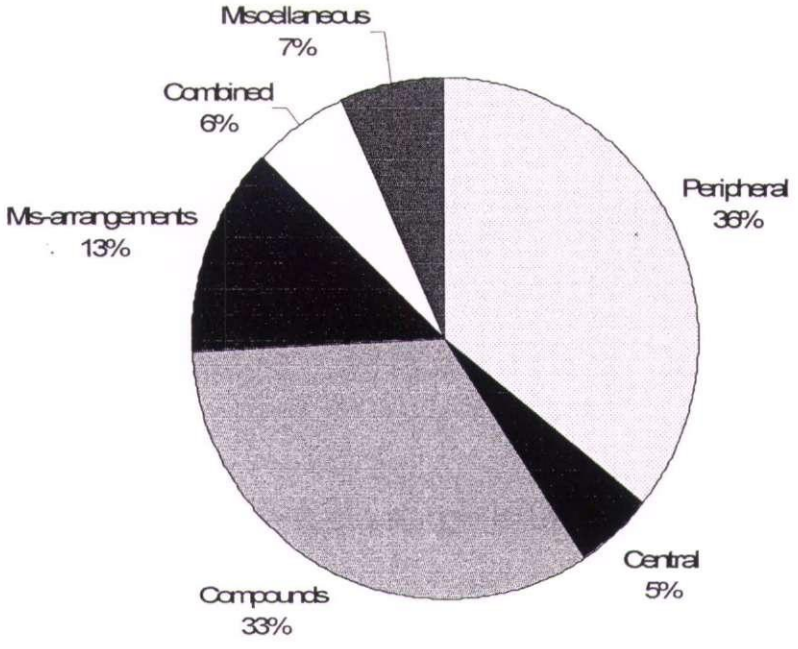


Figure 5.2 Pie chart showing relative contribution of different types of cilial abnormality in all patients

The incidence of each of the different types of abnormality was not normally distributed. The most prevalent type of abnormality in both smokers and non-smokers was the peripheral tubular defect (missing or extra peripheral tubules), followed by the compound (more than one axoneme surrounded by a single membrane). Least common were the central defects (missing or extra central tubules). Examples of these ultrastructural abnormalities are shown in Plates 8 - 12.

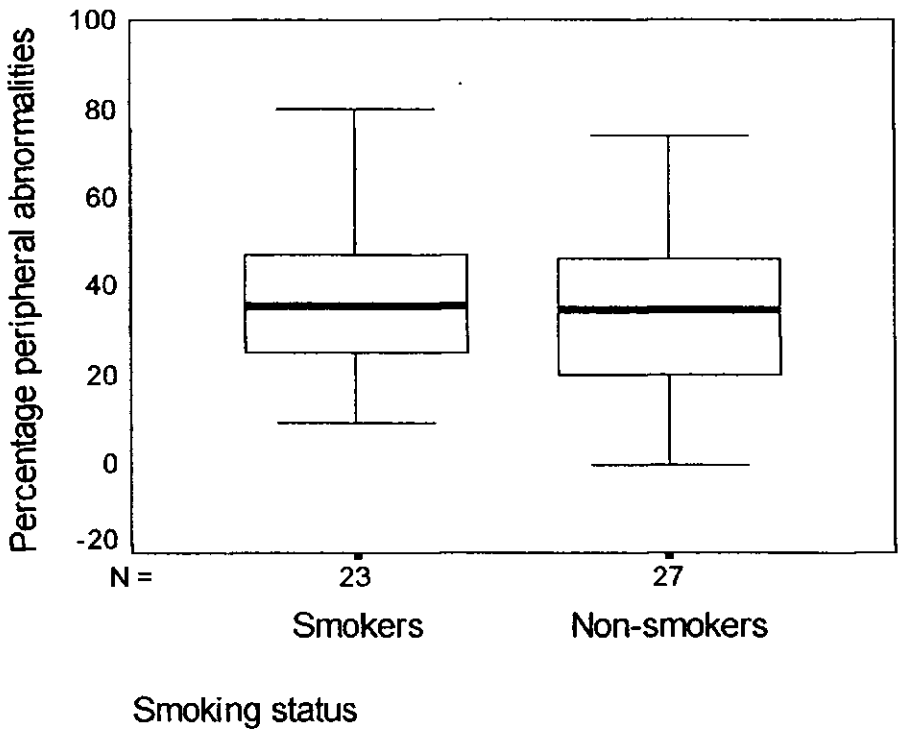


Figure 5.3 Box-plot showing proportion of abnormal cilia that were peripheral abnormalities in smokers and non-smokers. There was no significant difference between the two groups (p = 0.4, independent samples t-test).

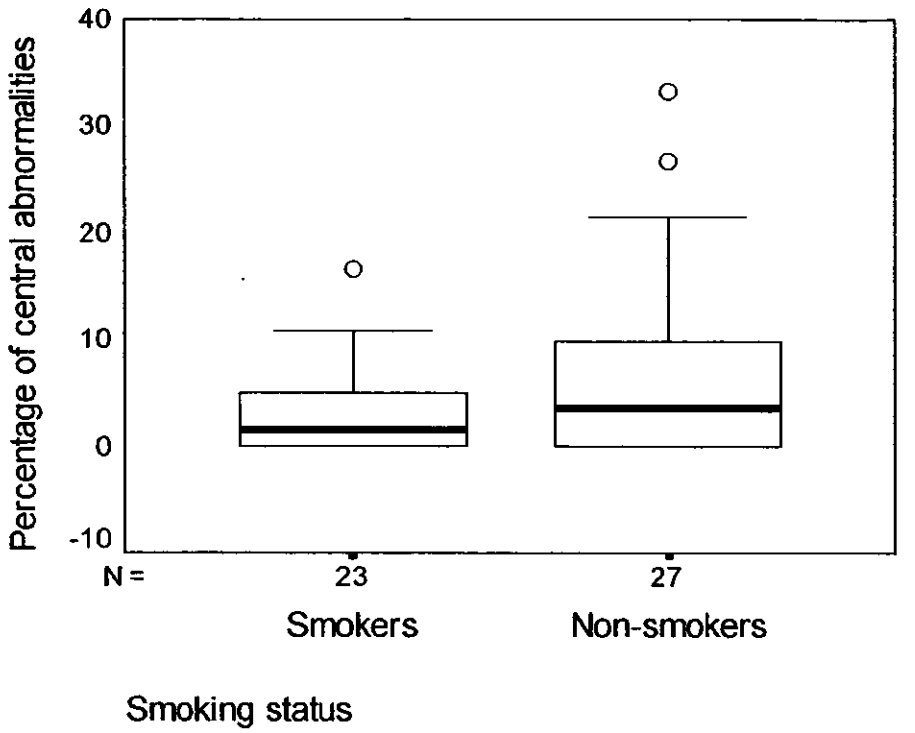


Figure 5.4 Box-plot showing proportion of abnormal cilia that exhibited central abnormalities in smokers and non-smokers. There was no significant difference between the two groups (p = 0.36, independent samples t-test).

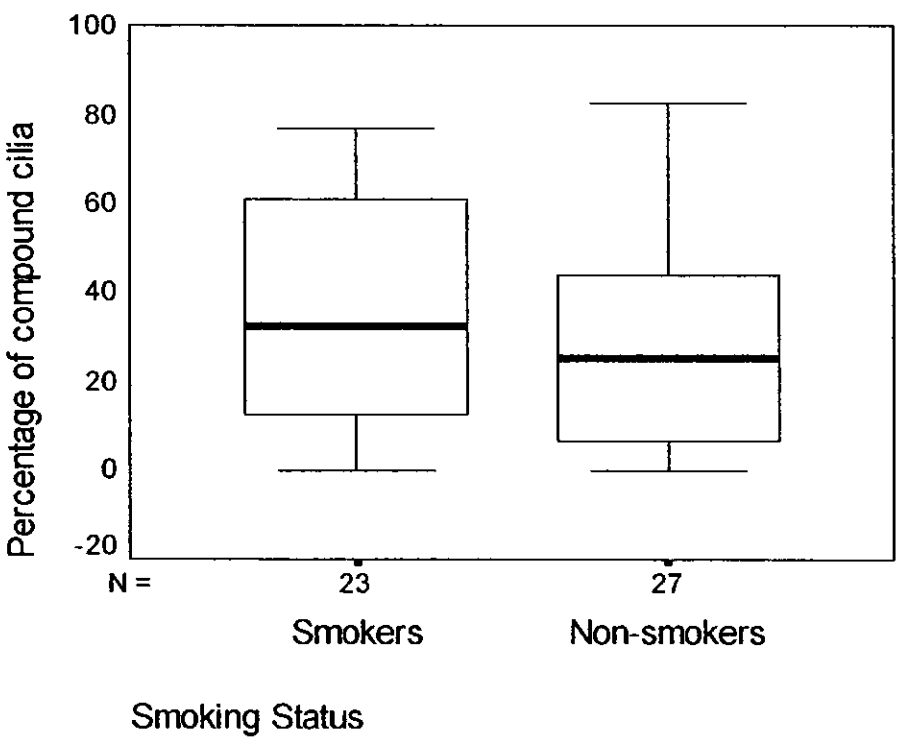


Figure 5.5 Box-plot showing proportion of abnormal cilia that were compound cilia in smokers and non-smokers. There was no significant difference between the two

groups (p = 0.43, independent samples t-test).

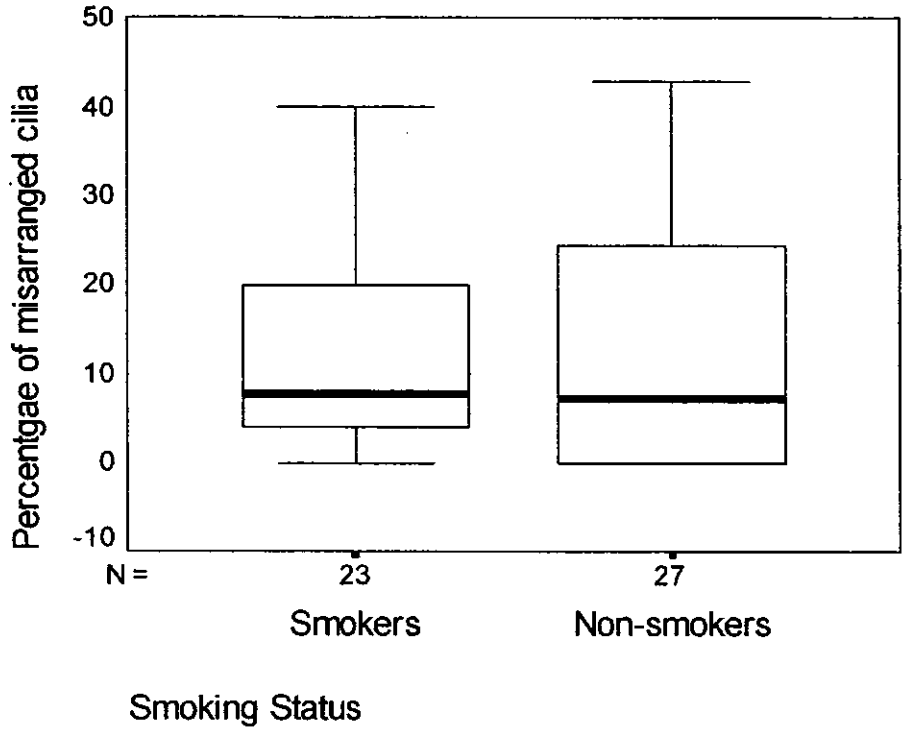


Figure 5.6 Box-plot showing proportion of abnormal cilia that exhibited missarrangement of microtubules in smokers and non-smokers. There was no significant difference between the two groups (p = 0.91, independent samples t-test).

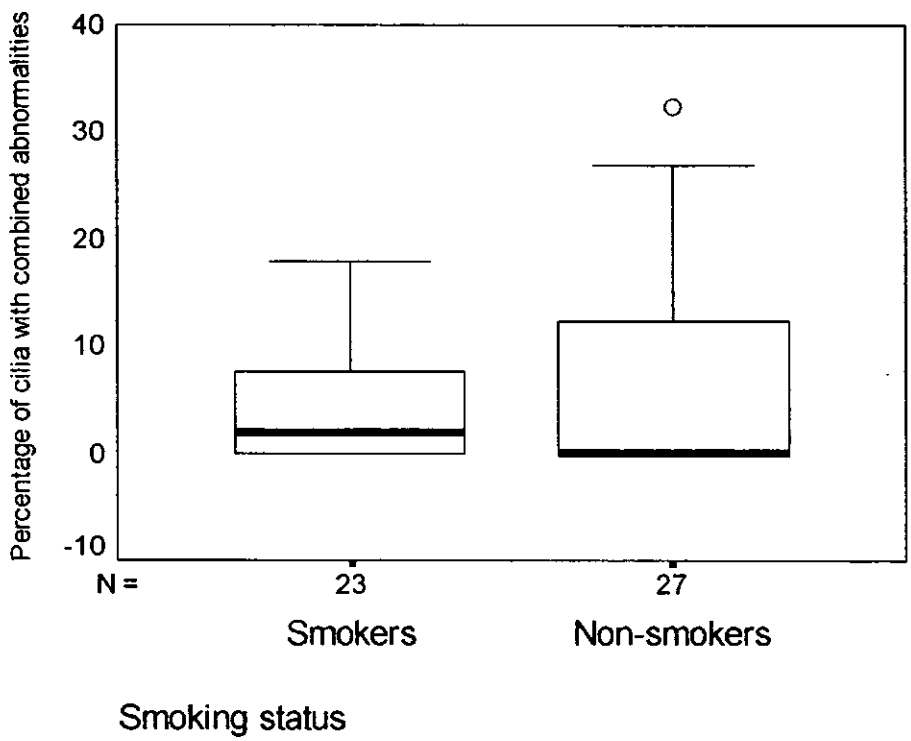


Figure 5.7 Box-plot showing proportion of abnormal cilia with combined abnormalities in smokers and non-smokers. There was no significant difference

between the two groups (p = 0.31, independent samples t-test).

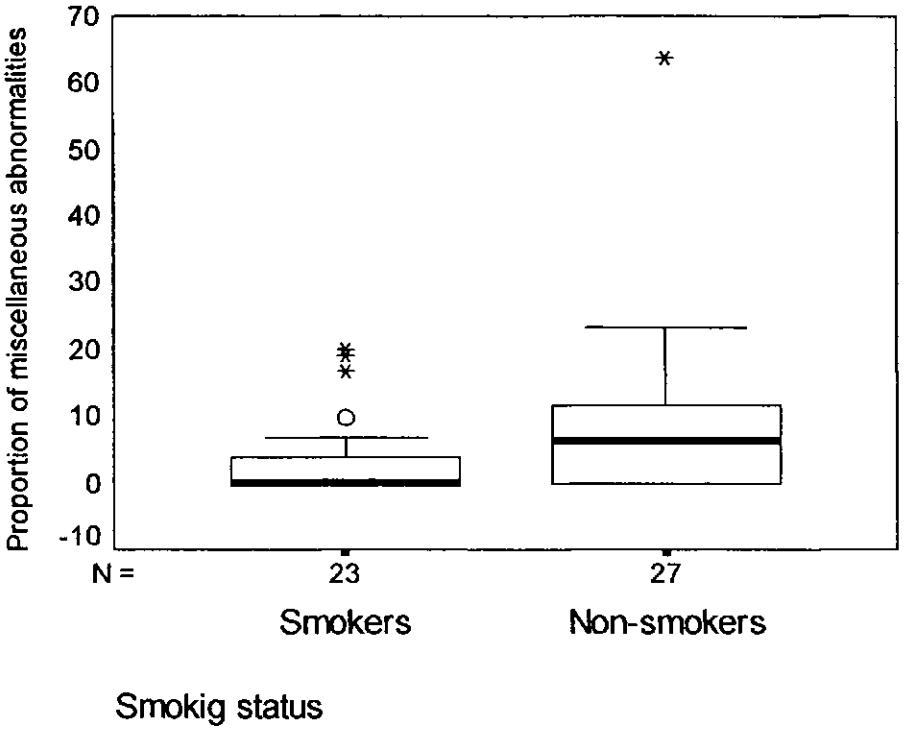


Figure 5.8 Box-plot showing proportion of abnormal cilia with miscellaneous abnormalities in smokers and non-smokers. There was no significant difference between the two groups (p = 0.03, independent samples t-test).

Plate 6

A Transmission electron micrograph showing transverse cross-sections of cilia (Ci), interspersed with microvilli (Mi). In the cross-section of the cell at the bottom of the image numerous basal feet of the cilia can be seen (indicated by black arrows).

This image is typical of that found in both smokers and non-smokers. The beds of cilia would be magnified further in order to facilitate detailed examination.

Plate 6

A Transmission electron micrograph showing transverse cross-sections of cilia (Ci), interspersed with microvilli (Mi). In the cross-section of the cell at the bottom of the image numerous basal feet of the cilia can be seen (indicated by black arrows).

This image is typical of that found in both smokers and non-smokers. The beds of cilia would be magnified further in order to facilitate detailed examination.

A

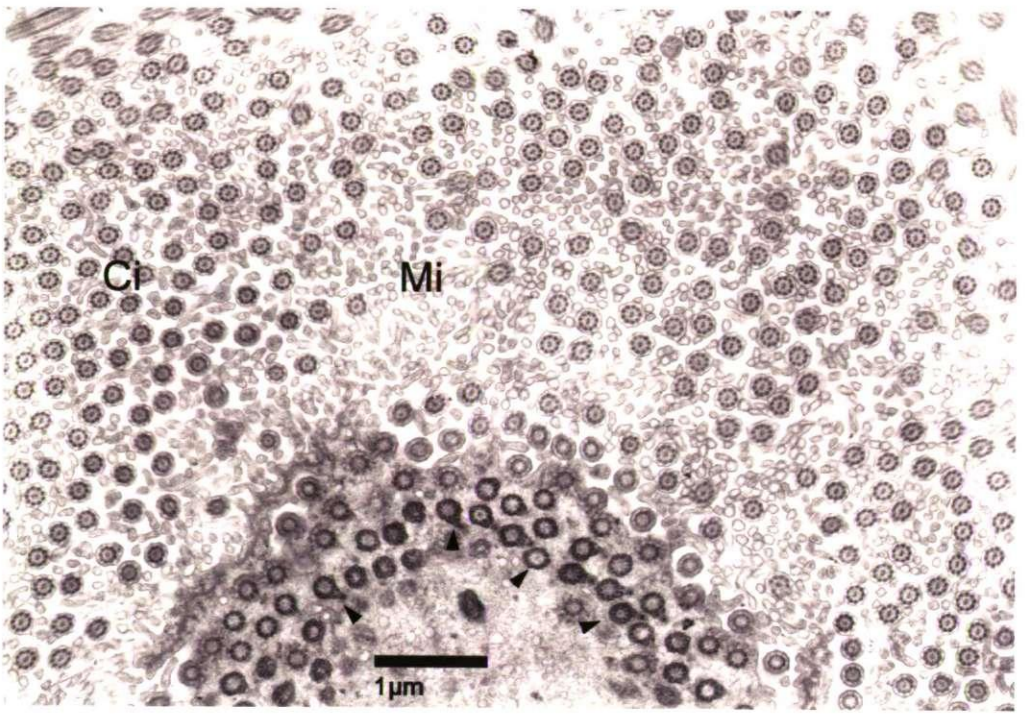


Plate 7

A Transmission electron micrograph of a compound cilium containing 7 axonemes (1). The compound is surrounded by several cilia with normal ultrastructure (2).

Patient: Female, smoker.

B Transmission electron micrograph of a compound cilium containing 4 axonemes (1). The surrounding cilia exhibit normal ultrastructure (2).

Patient: Male, non-smoker.

A

B

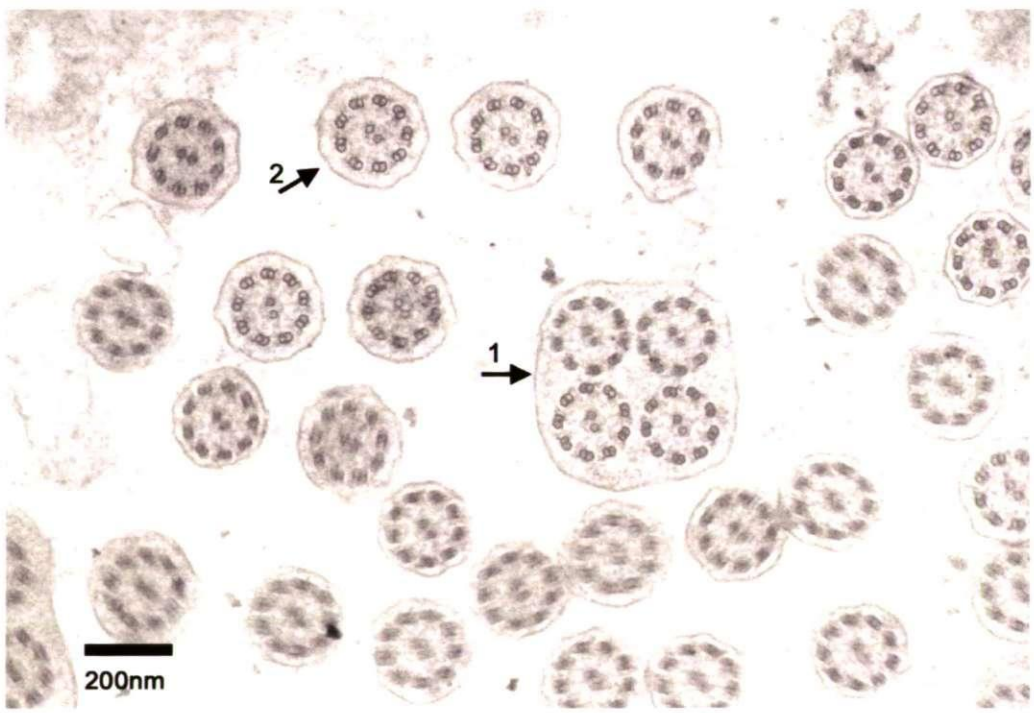


Plate 8

A Transmission electron micrograph showing various cilial abnormalities:

a Misshapen cilial membrane containing one complete axonemes and disorganised microtubules of possibly two others.

b Cilia with extra microtubule doublets

c Misarranged compound cilium

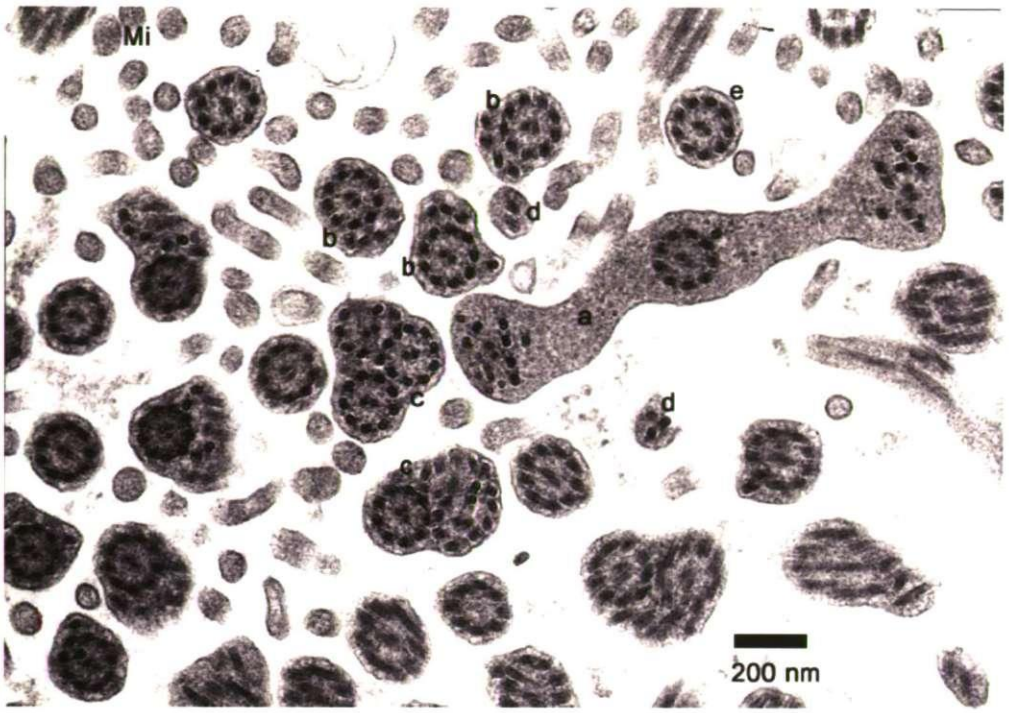
d Three microtubule doublets within a membrane

Patient: Female, non-smoker.

B Transmission electron micrograph of two compound cilia (1) surrounded by normal cilia (2).

Patient: Female, non-smoker (same patient as above).

A



B

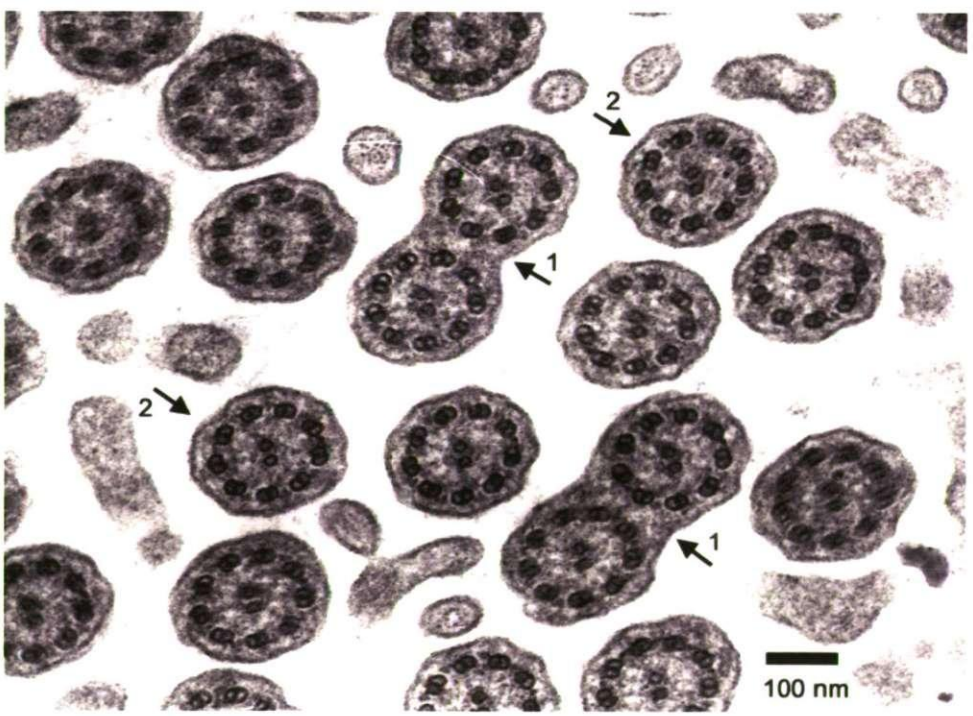


Plate 9

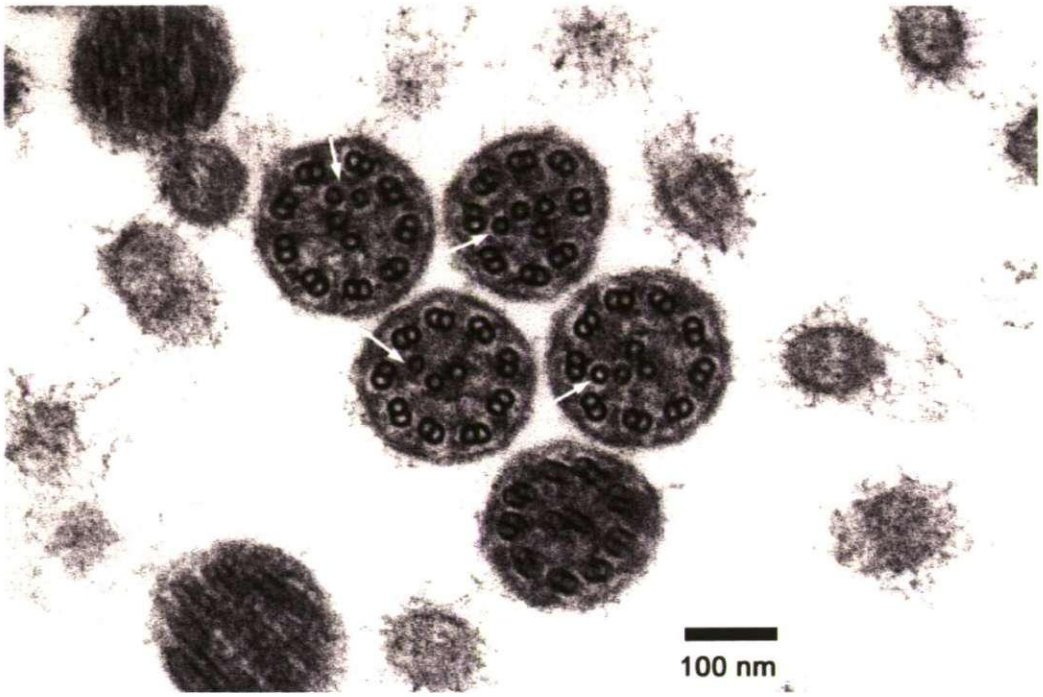
A Transmission electron micrograph of cilia containing extra central pairs (indicated by white arrows).

Patient: Female, non-smoker.

B Transmission electron micrograph showing a compound cilium with two extra microtubule doublets (1) and two cilia with missing peripheral microtubules (2).

Patient: female, non-smoker

A



B

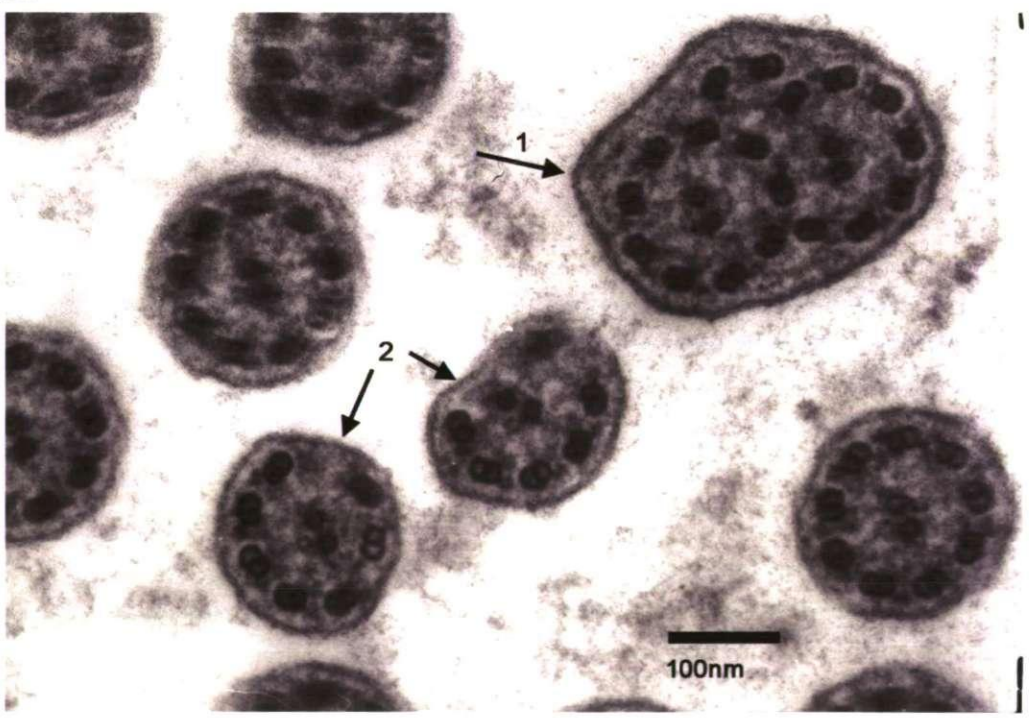


Plate 10

A Transmission electron micrograph of a 'hockey stick' cilium (1).

2 = normal cilium tip

Patient: male, smoker.

A

There was a slight upward trend in percentage cilial abnormality with increasing number of pack years in the smokers, as shown in Figure 5.9, but this was not statistically significant ($r = 0.35$, $p = 0.13$). There were no significant correlations between any individual type of abnormality and number of pack years (Table 5.3).

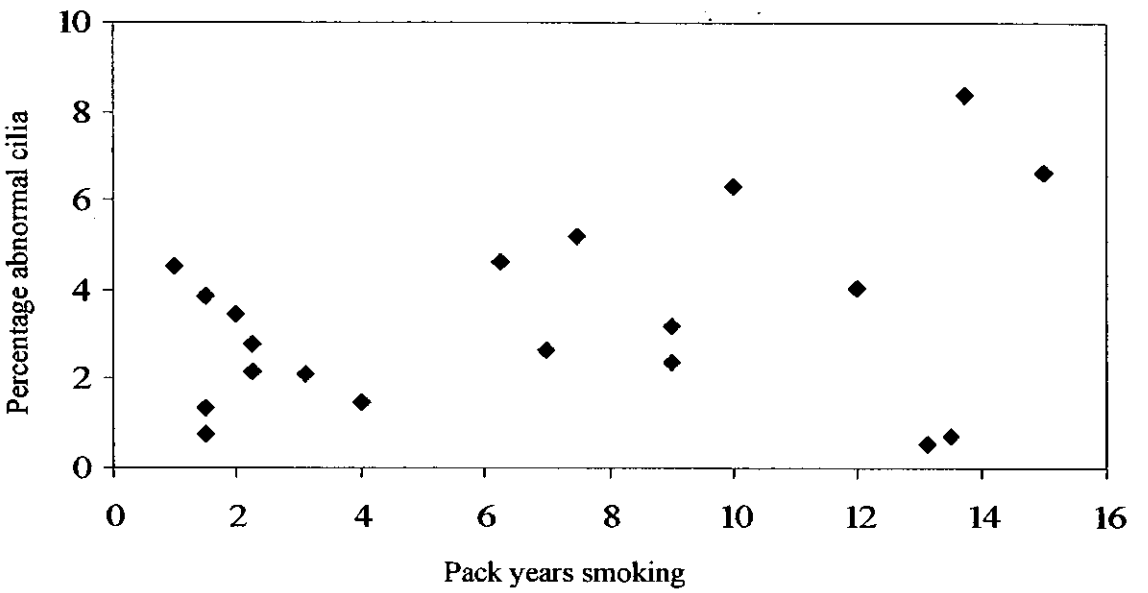


Figure 5.9 Relationship between number of pack years smoking and incidence of abnormal cilia in smokers (r = 0.35, p = 0.13, n = 20).

	r	p
Peripheral	0.38	0.10
Central	-0.15	0.52
Compound	-0.31	0.82
Misarranged	0.26	0.26
Combined	0.09	0.71
Miscellaneous	0.16	0.50

Table 5.2 Correlations between different types of cilial abnormalities and number of pack years smoking in asymptomatic smokers (n = 20).

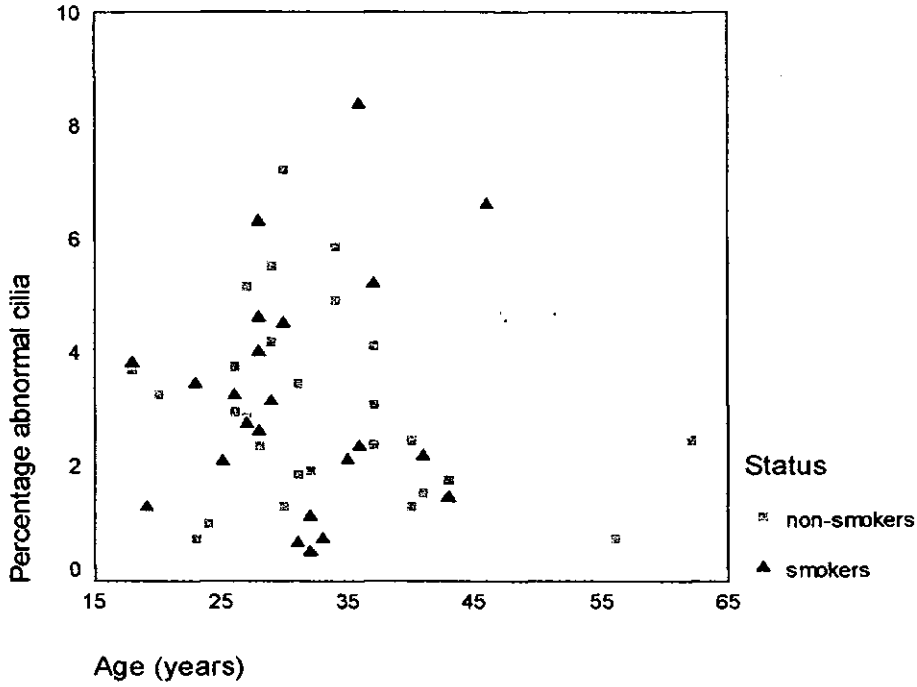


Figure 5.10 Relationship between incidence of abnormal cilia and age in asymptomatic smokers and non-smokers (r= -0.17, p= 0.31, n= 50).

There was no significant relationship between percentage abnormal cilia and age (r= -0.17, p= 0.31). There was a significant positive correlation between the incidence of peripheral abnormalities and age, conversely, a negative correlation was found between the incidence compound cilia and age (Table 5.4).

	R	p
Peripheral	0.36	0.03
Central	-0.007	0.97
Compound	-0.30	0.07
Misarranged	0.20	0.22
Combined	0.12	0.45
Miscellaneous	0.11	0.53

Table 5.4 Correlations between different types of cilial abnormalities and age in asymptomatic smokers and non-smokers (n= 50)

Further analysis revealed that the correlation between age and percentage peripheral abnormalities of cilia was found to be present only in the smokers ($r = 0.54$, $p = 0.03$ (percentage peripheral abnormalities = age x 0.025 – 0.97)).

Orientation

The method by which orientation was measured is illustrated in Plates 4 and 5. None of cilia could be classified as grossly disorientated (SD > 35°), there was no significant difference between the two groups (nested ANOVA, $p = 0.45$) and the distribution was similar ($p = 0.21$, Mann-Whitney test)

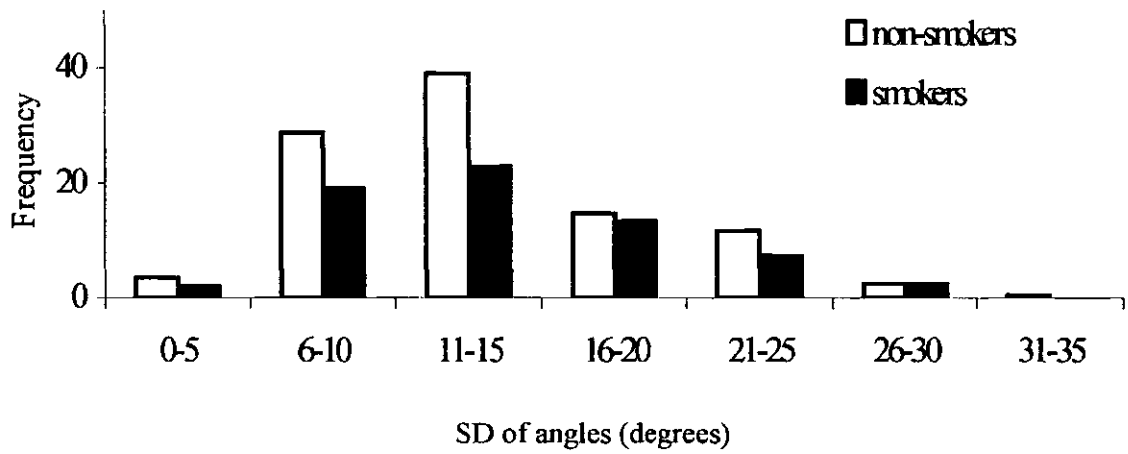


Figure 5.11 SD of angles of cilial alignment in asymptomatic smokers and non-smokers.

Although none of the cilia could be classified as grossly disorientated, there was a moderate correlation between the extent of disorientation and percentage abnormal cilia ($r = 0.50$, $p < 0.01$).

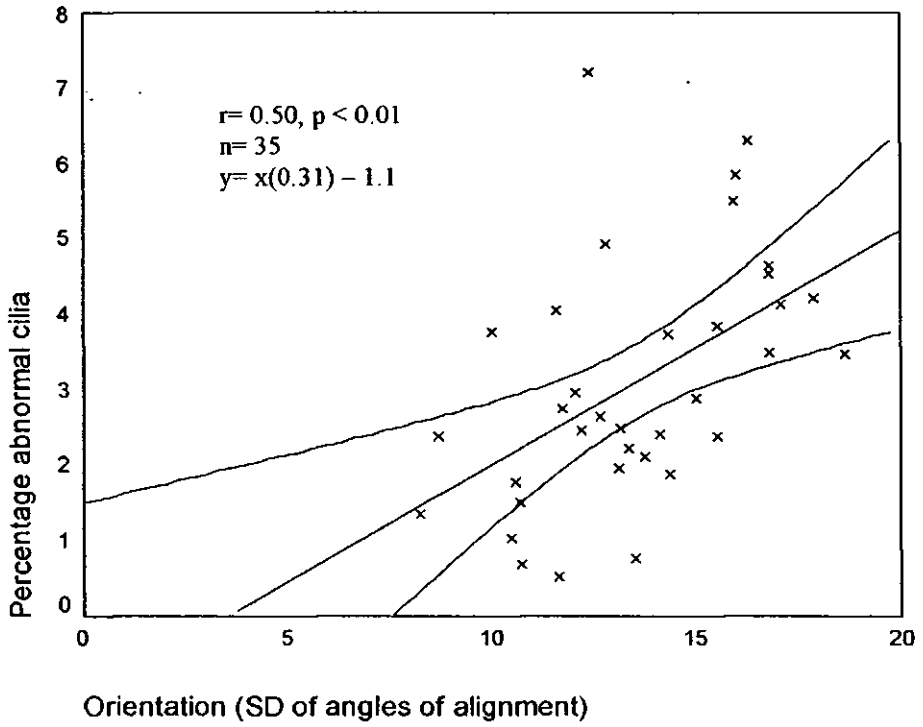


Figure 5.12 Relationship between orientation and incidence of abnormal cilia in asymptomatic smokers and non-smokers.

5.4 Discussion

There was no difference in the percentage of cilial abnormalities between asymptomatic smokers (3.21%) and non-smokers (3.05%). Nor was there any difference in the extent of disorientation between the two groups. A posterior power calculation revealed that this study of 50 patients could detect a minimum difference of 2% in cilial abnormalities between the two groups with 99% power. The probablity of a type II error was therefore just 1%.

The abnormalities found (Table 5.2) were polymorphic, which is characteristic of those that are acquired. Cilial abnormalities have been described in a variety of conditions including Primary ciliary dyskenesia (PCD) and related inherited disorders (Afzelius 1976) in which the critical importance of cilial ultrastructure to their function is obvious. These patients have a high proportion of specific abnormalities, which lead to impairment or complete loss of cilia beating, which results in poor or no mucus transport. Cilial abnormalities are also associated with chronic respiratory infection but seem to disappear once the infection is over (Carson et al., 1985). Disorientation of cilia (SD of angles of alignment > 35°) has also been previously described and in some cases of PCD is the only underlying cause of disease (Rayner et al., 1996). None of the patients in the present study had an abnormally high proportion of cilial abnormalities or disorientation. The amount of cilial abnormalities in healthy people is widely reported to be around 5%, with which the results of this study compare. The correlation between orientation and abnormalities is also consistent with previously published research.

The origins of abnormal cilia are unclear. It has been suggested that compounds and

tubular abnormalities may be formed during ciliogenesis and that compounds may also arise as a result of the fusion of pre-existing cilia. It is also believed that compounds may not in fact be pathologically altered cilia, but part of normal variation (Lungarella et al., 1983). The types of abnormal cilia found in this study were similar in the smokers and non-smokers. In this group of patients, the peripheral tubular anomalies were the most common of the abnormal cilia followed by compound. Other abnormalities including miss-arrangements and miscellaneous were also present in smaller numbers, and least common were the central pair anomalies. The present findings are contradictory to previous research which has suggested that smoking induces formation of abnormal cilia. The discrepancy may be explained by the different populations studied and the methodology used. Specifically, and in contrast to other studies, the focus in the present study was on asymptomatic patients only. The focus of previous studies was on symptomatic patients, including those with PCD, bronchitis, and chronic sputum production (Lungarella et al., 1983; Trevisani et al., 1992; Verra et al., 1995). This study was unique in its aim to study only asymptomatic patients in order to determine whether or not there are an increased number of cilial abnormalities in smokers prior to the onset of disease. Verra et al (1995) concluded that tobacco smoke induces cilial abnormalities after studying 37 adults with chronic sputum production, 13 were current smokers (of whom 6 had bronchiectasis and 7 had chronic bronchitis), 5 were ex-smokers (all with bronchiectasis), 19 were non-smokers (all but 1 had bronchiectasis) and there were just 5 healthy non-smokers. In addition several of their patients had an acute tracheobronchial infection (known to be associated with increased proportion of abnormal cilia (Carson et al., 1985)) at the time of sampling. We compared a much larger group of asymptomatic smokers and non-smokers, enabling a minimum difference of 2% to be detected between the two groups. This study was also carefully blinded to ensure no subjectivity during the scoring process, a point which is not

always stated in previous studies.

Despite the lack of a difference in the proportion of abnormal cilia between the smokers and non-smokers, there was a slight upward trend in abnormal cilia with increasing pack years of smoking. However, this trend was not found to be statistically significant. This may be because the mean number of pack years of the asymptomatic smokers was just 6.8 ± 4.9 years and there were very few at the higher end of this range. In order to further investigate the relationship between the number of pack years and abnormal cilia, more heavy smokers need to be included (the mean number of pack years in the asymptomatic smokers in one study was 22.62). In addition, the present study found a significant correlation between age and proportion of peripheral cilial abnormalities in the smokers only. However, the correlation was moderate and explained only 30% of the variation.

In summary, there was no difference in proportion of cilial abnormalities or orientation between smokers and non-smokers in this patient group - probably because they were all asymptomatic. This finding suggests that, prior to the onset of disease, smoking does not induce an increased proportion of abnormal cilia or disorientation. Consequently the increased proportion of abnormal cilia found in smokers with respiratory diseases, such as bronchitis, is more likely to arise as a result of (rather than causal to) the disease process itself. However, it should be noted that few heavy smokers were investigated in the present study and the correlation between age and peripheral cilial abnormalities in smokers suggests that heavier smokers (asymptomatic if possible) should be investigated to confirm that, in the absence of symptoms, smoking does not induce formation of abnormal cilia.

CHAPTER SIX

Abnormalities and abundance of cilia in critical illness

6 Abstract

Mucociliary clearance is known to be impaired in the critically ill. Abundance and ultrastructure of cilia are important determinants of efficient mucus clearance. Work in this area is very limited. Previous studies of cilia in critically ill patients are limited to just 3 days. The present study investigated ciliary abnormalities and abundance in patients whose length of stay varied from 1 to 28 days. There was a great variability in the incidence of abnormalities and abundance of cilia in these patients. None of the patients exhibited an incidence of abnormal cilia greater than 15%, the cut-off above which mucus transport rate is affected and no clear link with length of stay was found. There was diversity in the abundance of the cilia among the patients and a significant correlation between cilial abundance and admission APACHE II score was found, but of unknown importance.

6.1 Introduction

Mucociliary clearance is known to be impaired in the criticially ill. Konrad et al (1995). (Konrad., et al. 1995) measured bronchial mucus transport velocity (BTV) using technetium 99m- labelled albumin microspheres. In this study, the movement of the microspheres after deposit at the distal end of the right and left main bronchus was measured within three days of admission to the ICU and then daily for four days. The patients that went on to develop pulmonary complications were found to have a significantly slower BTV than those without: 0 (0-6.5) mm/min vs 3.5 (0-10.5) mm/ min in the left bronchus and 0 (0 – 3.0) mm/ min vs 4.7 (0 – 11.7) mm/ min in the right bronchus. The slower mucus transport was associated with the development of secretion retention and pneumonia. Slower BTV in intensive care patients is also associated with previous chronic cigarette smoking, pre-existing chronic bronchitis, suction induced lesions of the mucous membrane, ventilation with high concentrations of oxygen, colonization with potentially pathogenic microorganisms, infection with respiratory viruses, release of inflammatory mediators, and inadequate humidification of inspiratory gases. These factors were discussed in Chapter One.

Long term studies of the mucociliary transport system in intensive care patients are little known, with most of the work on cilia being carried out by a group led by Franz Konrad in Germany (Konrad et al., 1994; 1995). At present, the mechanism underlying impaired mucociliary transport in critically ill patients is unclear. In addition to investigating mucociliary transport, Konrad et al. (1995) have made an attempt to discover whether the observed reduced rate is associated with a loss of cilia or ultrastructural abnormalities. In a sample of 29 orally intubated patients BTV was measured with radio labelled

microspheres. Following this procedure bronchial biopsies were prepared both for scanning and transmission electron microscopy. Despite the semi-quantitative technique for determining the abundance of cilia in the biopsies, the scanning electron microscopy observations revealed that a loss of cilia was associated with reduced BTV. Conversely, the transmission electron microscopy revealed no apparent association of ultrastructural abnormalities nor disorientation and reduced BTV. However, no inferences relevant to long term intensive care or critical illness could be made since all measurements were completed within three days of admission to the ICU.

Chapter Five of this thesis presented the first large scale analysis of cilial abnormalities in 'healthy' adults, which can be used as a control group for the group of patients studied here. Presented here is the first, preliminary study of abnormalities and abundance of cilia in critically ill patients beyond three days of admission to the ICU.

6.2 Materials and Methods

6.2.1 Specimen collection

Samples of bronchial ciliated epithelium were collected by bronchoscopic biopsy and cytological brushing of the bronchi of critically ill patients receiving ventilation in the intensive care unit at Plymouth's Derriford hospital. Informed assent was obtained from relatives of the patients and samples were only taken when a bronchoscopy was clinically necessary. Small forceps were used to take biopsy samples from a visually normal area of mucosa beyond the first division of the main bronchus, where trauma due to suction catheter use would be unlikely. Cytological brushings were taken blind from the trachea using a cytology brush protected within a plastic sheath to avoid contamination from upper airway mucosa cells.

6.2.2 Electron microscopy

The cytological brushings were prepared for transmission electron microscopy as previously described in Chapter 5.

The biopsies were routinely processed for scanning electron microscopy and handled exclusively by Ross Kay (please refer to acknowledgements).

6.2.3 Ultrastructural (TEM) evaluation

Semi thin and ultrathin sections were cut using a Reichert ultramicrotome. The ciliated areas within each block of tissue were located by examining semi-thin sections (0.5 μm), stained with methylene blue, and observed using a light microscope. Once a ciliated area

was located several ultrathin sections (0.1 μm) were cut. Five different sets of ultrathin sections were cut, ensuring a large number of cilia would be analysed and that the same cilia could no be examined twice. Ultrathin sections were collected on copper grids and stained for 10 minutes with uranyl acetate and 15 minutes with lead citrate. They were then viewed using a Jeol 1200 transmission electron microsope operated at 80kV.

6.2.4 Abundance (SEM) evaluation

All measurements of cilial abundance were carried out by Ross Kay, who built on the quantitative technique described in Chapter 3. The method has been published (Kay et al., 2002) and full details can be found in his thesis (Kay, 2004).

6.2.5 Statistical analysis

The abnormality data were subject to logit transformation prior to analysis. Analysis of variance in percentage abnormal cilia between groups of patients was tested by unpaired t-tests. Relationships between duration of ICU stay and percentage abnormal cilia and abundance were explored using simple linear regression.

6.3 Results

Bronchial brushings and biopsies were taken from thirteen men and seven women. The patients included eleven smokers and seven non-smokers. Clinical characteristics of the patients were diverse, diagnoses included but were not limited to pneumonia, head injuries, hepatic failure, and AAA repair. A summary of the patients details are shown in Table 6.1.

	age (years)	duration of stay (days)	APACHE II score*
mean	61	10.3	17.8
standard deviation	18	7.6	6.5
median	62	8.0	18.0
range	22 - 84	1 - 29	6.0 - 27.0

Table 6.1 Summary of ICU patient details (n= 20)

*APACHE II score: predicts mortality, based on a combination of physiological variables including vital signs, haematology and chemistry.

Neither the men and women nor smokers and non-smokers differed significantly with respect to any of the above measures.

6.3.1 Ultrastructure (TEM)

Several of the samples were found to contain a large number of cilia with missing central tubules. Where this particular abnormality occurred in a large number it tended to be very focal in nature, appearing to affect individual cells only. These cells were also usually on their own and not associated with any other tissue. For this reason, the results have been this specific abnormality was excluded from analysis, since the results may be misleading. These cilia were frequently distinguished by the lack of a single central tubule and/ or incomplete peripheral tubules. This was often also associated with a lack of membrane surrounding the cilia. The significance of this specific abnormality is considered further in the discussion.

The abnormalities were more diverse than those found in the study of cilial abnormalities in asymptomatic smokers and non-smokers and were therefore classified into slightly different groups (Table 6.2). The most prevalent abnormality was the compound (28.2% of all abnormalities) followed by missing central tubules (26.1% of total abnormalities). The peripheral tubular defects accounted for 18.6% of all abnormalities. The least common of the abnormalities were those involving the central pair in the previous study, but since the majority of missing central pairs in this study were associated with autolysis in the present study they were excluded from subsequent analysis. Still, *extra* central pairs were included however, and these were one of the least common abnormalities in this study (1.3% of total abnormalities).

None of the following analyses include the missing central pair abnormality.

Once again the percentage cilial abnormalities were not normally distributed and were therefore subject to a logit transformation before analysis. Excluding the loss of central tubule anomaly, there were 2.7 ± 1.3% abnormal cilia in these patients.

The incidence of cilial abnormalities was 2.5% in the non-smokers and 3.0% in the smokers. Once again, there was no statistically significant difference between these two groups of patients ($p= 0.43$, unpaired t-test).

Type of abnormality		Percentage of total abnormalities*	
Central complex	Missing tubules	26.1 (not included in analysis)	
	Extra tubules	1.3	
Peripheral tubular	(including both missing and extra tubules)	18.6	
Compound	Normal ultrastructure	22.9	(total compounds: **28.2**)
	Abnormal number of tubules	4.1	
	Misarrangement	1.2	
Misarrangements		8.9	
Membrane		4.0	
Combined	Central + peripheral	5.9	
	Translocation	5.1	
	Central and/ or peripheral + misarrangement	1.3	
Other (misc)	'hockey stick'	0.3	
	Central + peripheral + misarrangement	1.3	
	Periciliary sheath	0.3	

Table 6.2 Prevalence of the different types of cilial abnormality in ICU patients

*Total numbers of each abnormality in all patients expressed as a percentage of total number of all abnormalities in all patients.

There was no correlation between the duration of ICU stay and percentage cilial abnormalities as shown in Figure 6.1. There was again no association between abnormalities and age as shown in Figure 6.2.

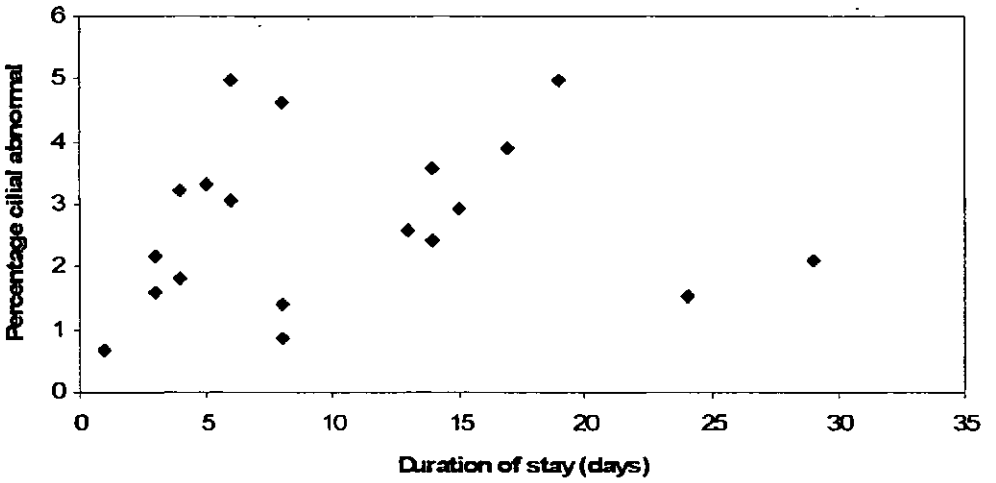


Figure 6.1 Relationship between duration of stay in the ICU and percentage cilial abnormalities in critically ill patients (r= 0.12, p= 0.58, n= 20)

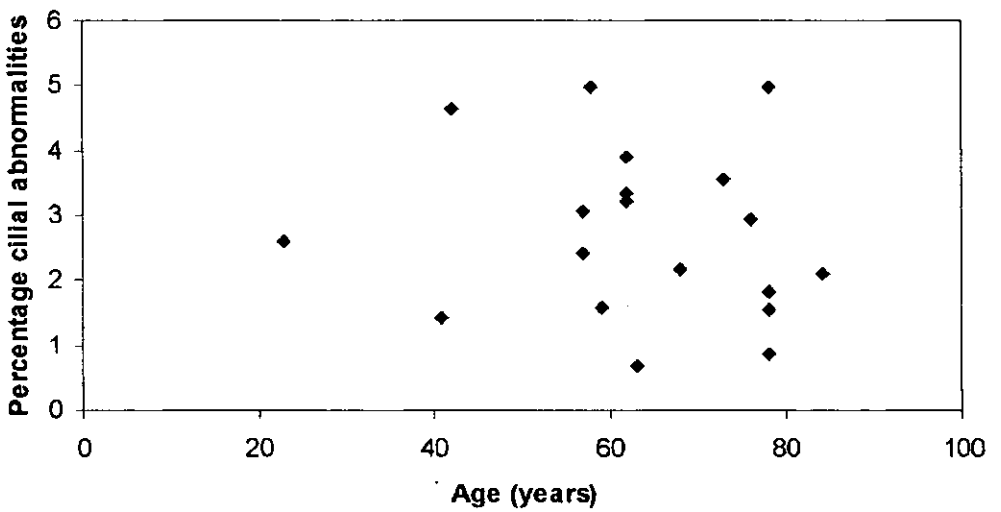


Figure 6.2 Relationship between age and percentage cilial abnormalities in ICU patients (r= -0.12, p= 0.61, n= 20)

Plate 11

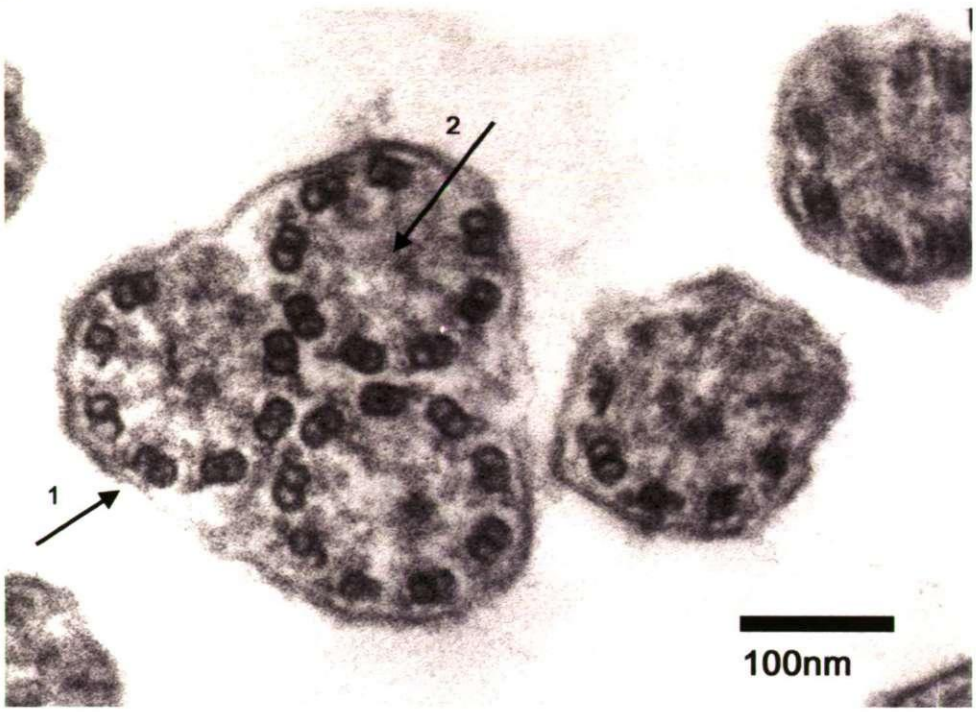
A Transmission electron micrograph of a compound cilium (1) – the axonemes are also missing central microtubules (2).

Patient
Gender: **Female**
Age: **68**
Apache II score: **19**
Duration of ICU stay at time of sample: **3 days**
Smoking status: **Non-smoker**

B Transmission electron micrograph of a compound cilium (1) – one of the axonemes is misshapen. Normal cilia (2).

Patient
Gender: **Female**
Age: **57**
Apache II score: **12**
Duration of ICU stay at time of sample: **14 days**
Smoking status: **Smoker**

A



B

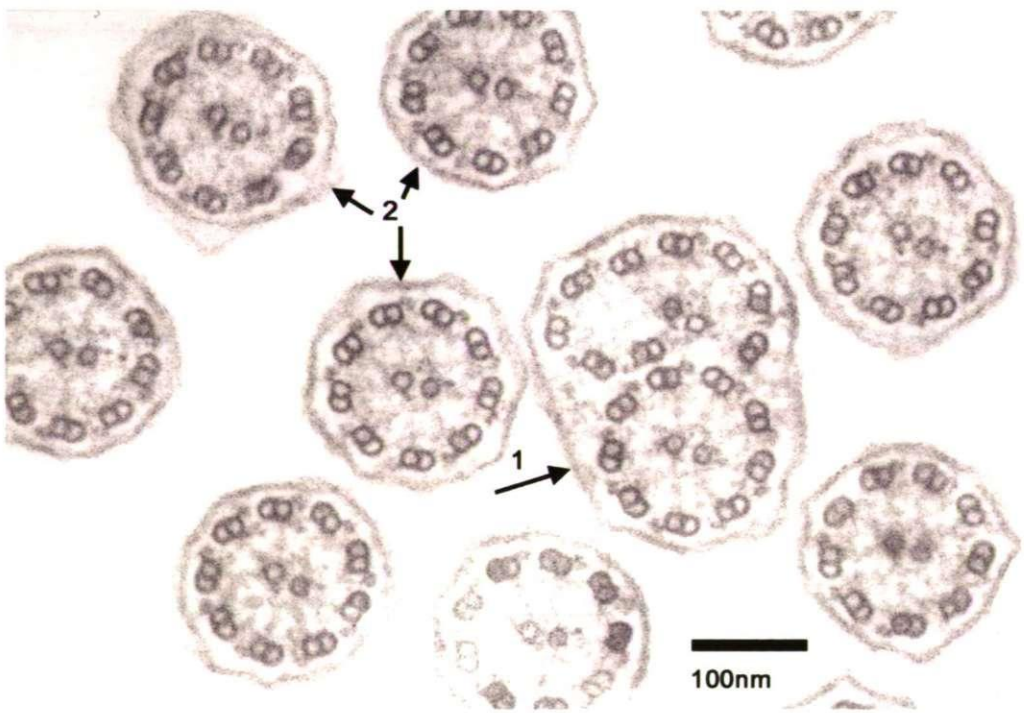


Plate 12

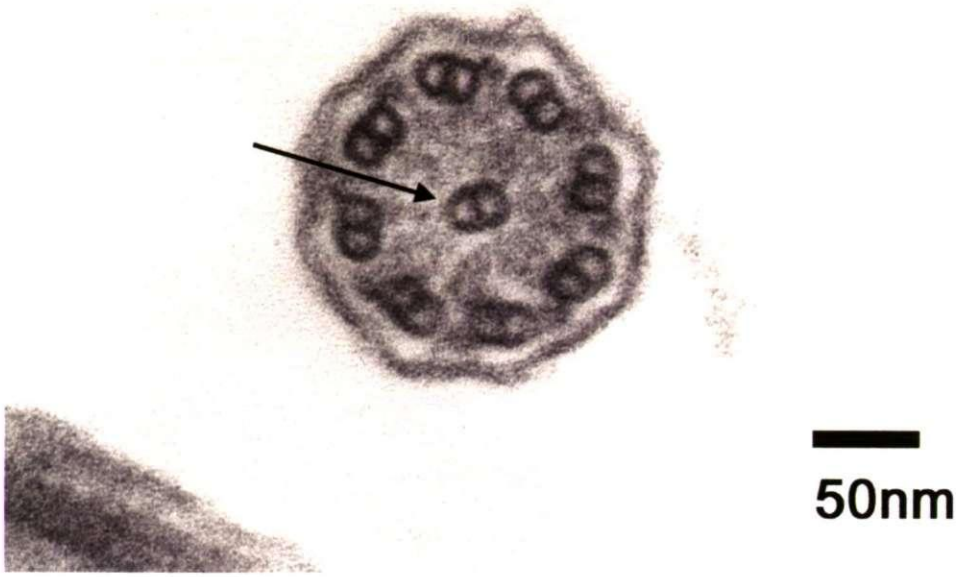
A Transmission electron micrograph of a 'transposition' defect. The central pair of microtubules appears to have been replaced by a peripheral doublet as indicated by the black arrow.

Patient
Gender: **Male**
Age: **62**
Apache II score: **15**
Duration of ICU stay at time of sample: **4 days**
Smoking status: **Smoker**

B Transmission electron micrograph of a cilium with the transposition defect (1, 2). Normal cilium (3).

Patient
Gender: **Male**
Age: **62**
Apache II score: **15**
Duration of ICU stay at time of sample: **6 days**
Smoking status: **Smoker**

A



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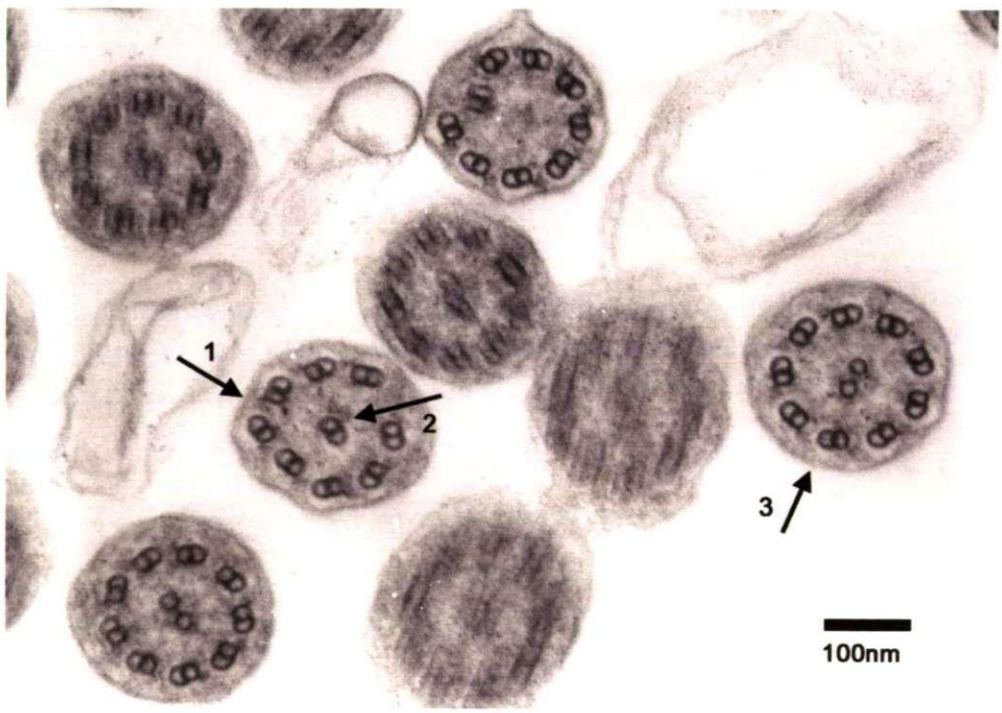


Plate 13

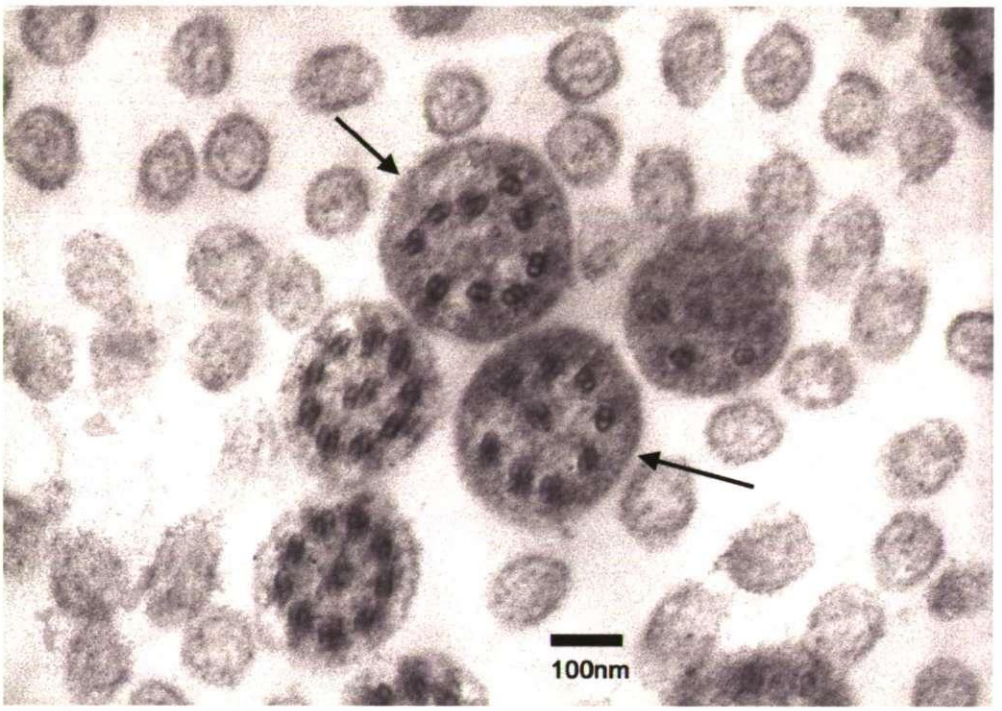
A Transmission electron micrograph showing cilia with expanded membranes and disorganised microtubules.

Patient
Gender: **Female**
Age: **41**
Apache II score: **8**
Duration of ICU stay at time of sample: **8 days**
Smoking status: **Non-smoker**

B Transmission electron micrograph of intracytoplasmic cilia (1).

Patient
Gender: **Female**
Age: **57**
Apache II score: **12**
Duration of ICU stay at time of sample: **14 days**
Smoking status: **Smoker**

A



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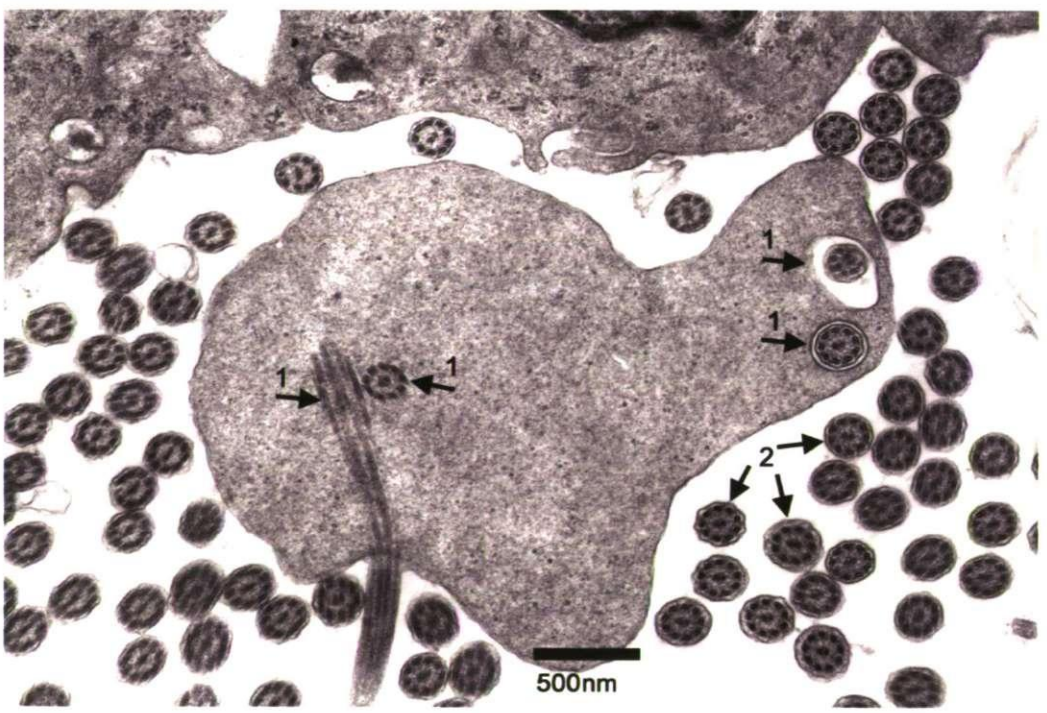


Plate 14

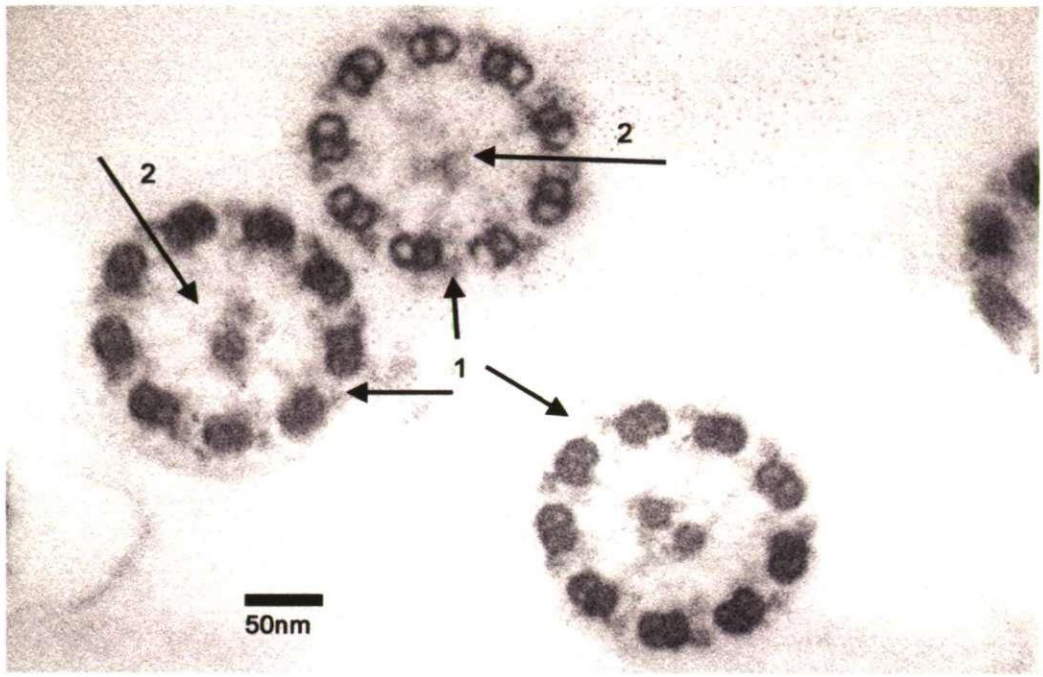
A Transmission electron micrograph of cilia with degraded membranes (1) and missing central microtubules (2).

Patient
Gender: **Female**
Age: **41**
Apache II score: **8**
Duration of ICU stay at time of sample: **8 days**
Smoking status: **Non-smoker**

B Transmission electron micrograph of cilia with missing central pair (1), extra central pair (2), and missing single central microtubule (3).

Patient
Gender: **Female**
Age: **76**
Apache II score: **24**
Duration of ICU stay at time of sample: **15 days**
Smoking status: **Non-smoker**

A



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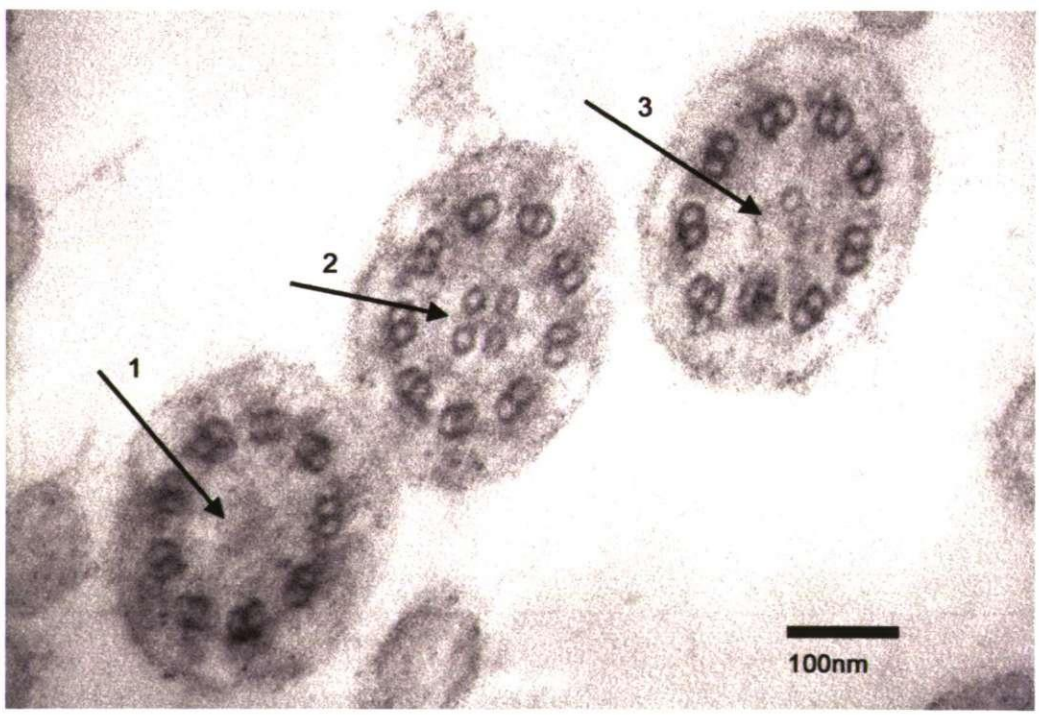


Plate 15

A Transmission electron micrograph of cilia, membranes intact, with missing central microtubules (indicated by black arrows).

Patient
Gender: **Female**
Age: **57**
Apache II score: **12**
Duration of ICU stay at time of sample: **12 days**
Smoking status: **Smoker**

B Transmission electron micrograph of a cilium with a missing central pair of microtubules (1). Normal cilia (2).

Patient
Gender: **Female**
Age: **57**
Apache II score: **12**
Duration of ICU stay at time of sample: **12 days**
Smoking status: **Smoker**

A

B

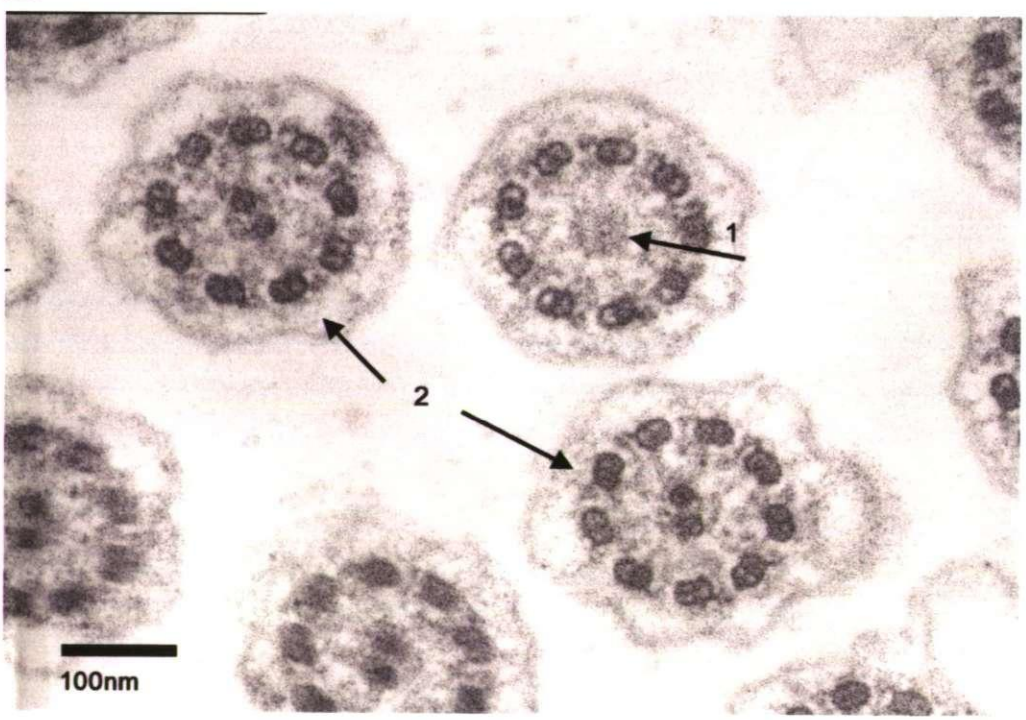


Plate 16

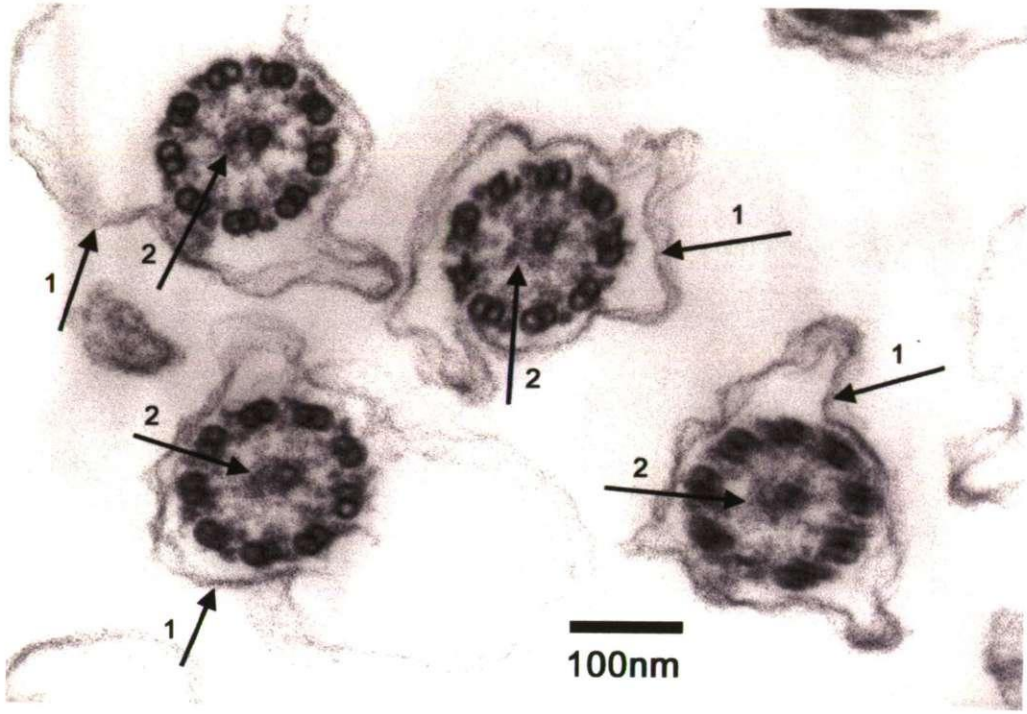
A Transmission electron micrograph of cilia with expanded membranes (1) and missing central microtubules (2).

Patient
Gender: **Female**
Age: **68**
Apache II score: **19**
Duration of ICU stay at time of sample: **3 days**
Smoking status: **Non-smoker**

B Transmission electron micrograph of compound cilium (1) with one of the axonemes lacking in a central pair of microtubules (2). Two other cilia have missing central pair microtubules (3) and degenerated or poorly stained membranes. Normal cilium with (4) degenerated or poorly stained membrane.

Patient
Gender: **Female**
Age: **76**
Apache II score: **24**
Duration of ICU stay at time of sample: **20 days**
Smoking status: **Non-smoker**

A



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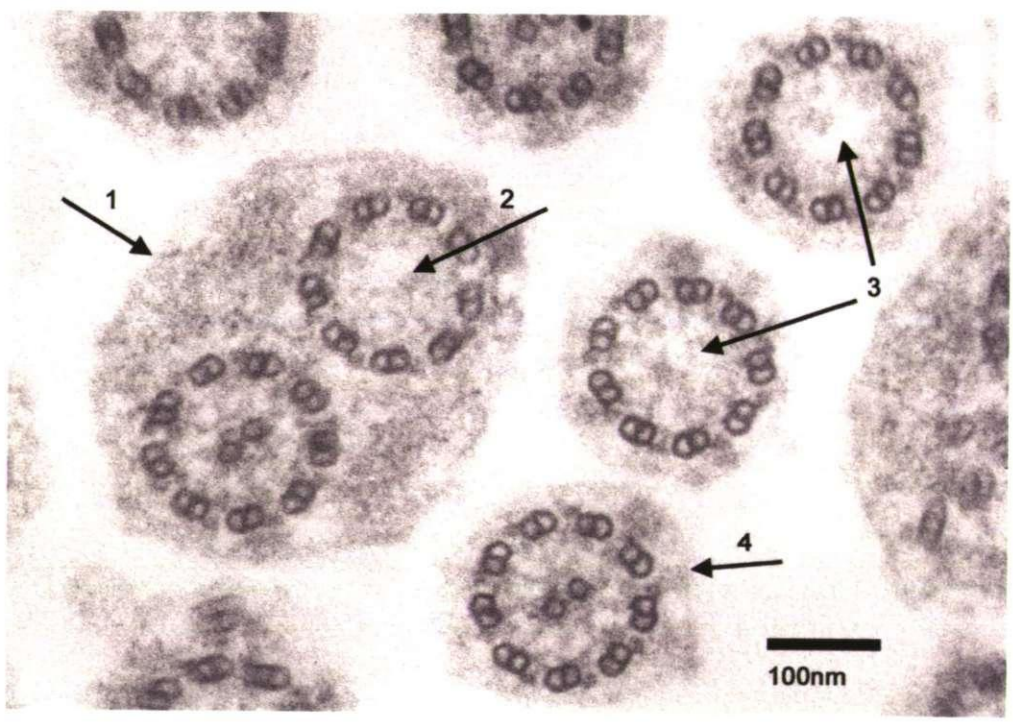

6.3.2 *Abundance*

Of 22 biopsy samples, abundance measurements were obtained from 15. There was a wide variety in the abundance of cilia among the samples as shown in table 6.3.

n	15
Mean	29.8
Median	1.0
Standard deviation	40.5
Range	97.3
Min	0
Max	97.3

Table 6.3 Percentage cilial abundance in bronchial biopsies from ICU patients.

There was no relationship between the percentage cilial abundance and percentage abnormal cilia, nor between duration of ICU stay and abundance (Figure 6.3).

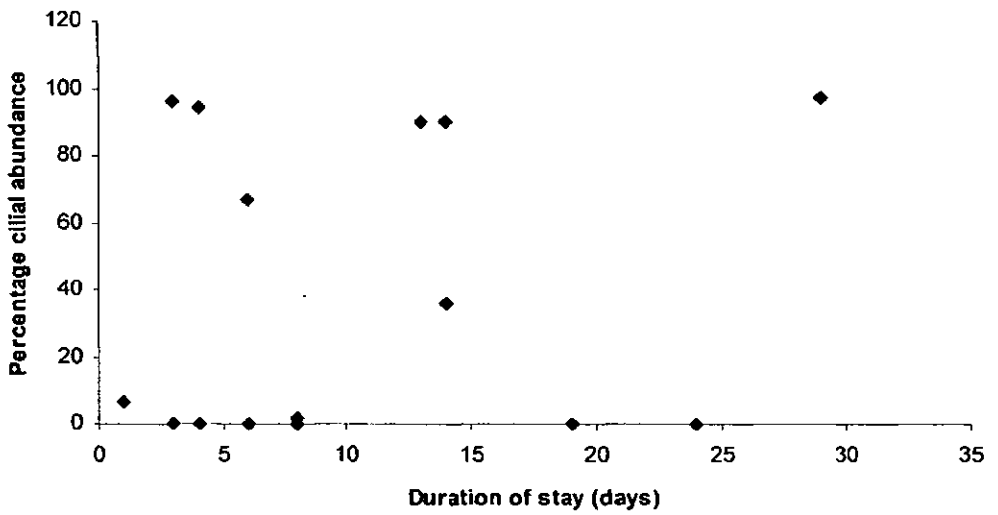

Figure 6.3 Variance of cilial abundance with duration of ICU stay in bronchial biopsies from ICU patients (r= 0.16, p= 0.57, n= 15)

There was a significant correlation between the admission APACHE II score and cilial abundance (r= -0.65, p=0.02).

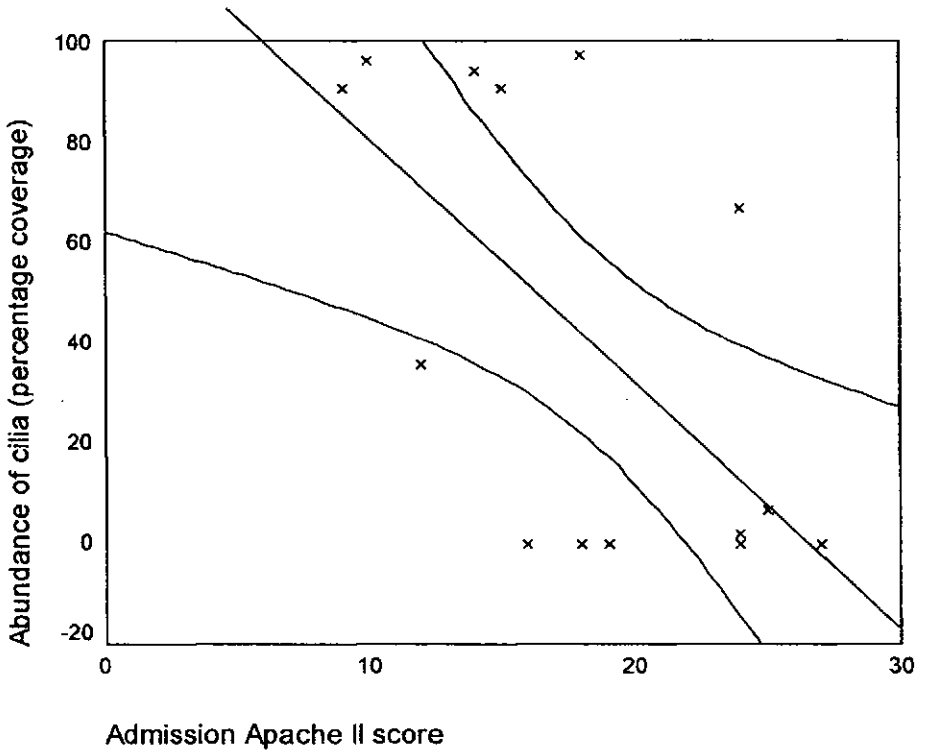

Figure 6.4 Variance of cilial abundance with APACHE II score in bronchial biopsies from ICU patients (n= 15)

6.4 Discussion

Presented here for the first time are data on cilial ultrastructure and abundance in critically ill patients beyond three days of admission to the ICU. The data reported here represent the preliminary findings of an ongoing study. The only inclusion criterion for the patients was that a bronchoscopy was required, which meant that patients with existing respiratory complications such as pneumonia were included. In fact, the patients had a variety of diagnoses and microscopy results. This and the small patient number are very important limitations and restrict the conclusions that can be drawn from this study.

The samples collected from the patients included in this study exhibited great variability. They contained on average 4.9% abnormal cilia. This was slightly higher than that found in the healthy smokers and non-smokers.

The types of abnormality found are of particular interest though questionable importance. The central complex abnormality described in the results was particularly focal, not just to a general area but limited to individual, usually lone cells. Frequently, these cells lone cells were not associated with any other tissue. This abnormality was often combined with incomplete B microtubules and a degraded cilial membrane. Due to the highly focal nature of this abnormality, it was not included in the final analysis of results. It is unlikely that this is a preparation artefact, since it was found in only a few cells in any given sample and not at all in some samples, all of which were processed in the same way.

The significance of central complex (CC) abnormalities have been discussed previously (Chapter 1), particularly in the context of being congenital and associated with PCD. In

one recent report CCs were the main abnormality in a group of patients with chronic respiratory infection (Tamalet et al., 2001). Acquired CC abnormalities are less commonly described, and in such reports usually only a few cilia are affected (Carson et al., 1985), (Lungarella et al., 1983). It is likely that the CC abnormalities described in the present study represent degradation due to autolysis before fixation and for this reason were excluded from further analyses. Autolysis is usually morphologically characterised by cell shrinkage and blebbing of the nuclear envelope. Unfortunately, the nature of the sections was such that the affected cilia did not tend to be associated with any other tissue, making it difficult to assess these changes. Specific autolytic changes associated with cilia are not commonly described in the literature and little research describes this particular abnormality. It is thought that a 'lot of autolysis of cilia goes on in life'(Fox, Bull et al. 1983) and at least one other paper ascribes the abnormality we have described here to autolysis. Afzelius described what he termed the 'artifactual absence' of central microtubules in a specific sample and was able to trace this anomaly to 'pronounced autolytic changes post-mortem'. In contrast, Lee et al (1987) studied ultrastructure and function of respiratory cilia in patients up to 16 hours post-mortem and found that although cell autolysis was evident, the cilia displayed normal ultrastructure and motility. Van der Baan et al (1987) carried out a quantitative analysis of cilial ultrastructure in healthy individuals and in PCD patients. They described an abnormal number of central tubules associated with an incomplete B microtubule along with frequent degeneration of the membrane, just as the present study has done. In agreement with this study, this combination of anomalies leads to the conclusion that this is indeed a feature of autolysing cilia. Conversely, others believe that this anomaly is specific to infection (Sturgess et al, 1984). Carson et al (1985) describe 'microtubular discontinuities' associated with chronic respiratory infection. These cilia were characterised by incomplete peripheral microtubules

and frequently missing central tubules. However, evidently in their study the cilial membranes were well preserved and as such the CC abnormality was ascribed to the process of infection rather than to autolysis. Finally, Rutland et al (1982) present an interesting case report, describing almost half the cilia in a sample from a patient with chronic respiratory disease as lacking in central pairs. Examination of a subsequent sample taken six weeks later, however, revealed no evidence of this defect. Again the cilial membranes were intact in this case and the anomaly was believed to be a consequence of local inflammation following infection. With the combination of missing central tubules, incomplete peripheral tubules, and degeneration of the cilial membrane, which characterised the abnormality in our samples, it seems reasonable to conclude that this represents autolysis of the cilia. Since the handling of the samples was identical it may be that during the sampling technique itself, there was some contamination with already dead tissue.

This study has again confirmed the highly focal nature of cilial abnormalities. This finding has been documented previously by Fox et al (1983) in a study designed to assess the distribution and significance of cilial abnormalities. They noted that there were considerable variations in the incidence of abnormalities seen in different biopsies taken from different sites in the same patient. They suggested that in order to overcome these difficulties, a large number of cilia should be counted. They recommended a minimum of five hundred. At least this many in these samples where possible, and in several cases many more were counted in the present study. However, as seen in this study, even when a large number of say five hundred cilia are counted, if the abnormalities are very focal in nature, a misleading result may still be obtained. This furthers the need for rigorous blinding techniques in any study of cilial abnormalities. This point is not always stated in

some studies but is utterly essential to ensure unconditional objectivity when counting. Perhaps the only way to ensure absolute accuracy would be to scan the entire sample and count every single cilium. Such an approach needn't be as daunting or laborious as it might first appear since the process could be efficiently speeded up by the use of a digital camera attached to the microscope. This would enable contiguous images to be collected covering the entire section. The cilia could then be counted automatically by a computer program, while the investigator only had to look for and count the abnormalities.

An increased number of abnormal cilia is likely to ultimately reduce mucus transport as previously described. This in turn predisposes to infection. Microorganisms are also known to synthesise and release factors capable of impairing CBF and loss of mucosal integrity. The correlation between an increased incidence of abnormality and reduced cilial motility was recently confirmed in non-PCD patients by Willems and Jorissen (2000). A significant decrease in CBF was associated with an incidence of abnormalities greater than 15%. Excluding the CC abnormalities, the compound cilium was found to be the most prevalent abnormality among the samples in the present study. It has been suggested that an increased number of compounds is associated with infection and smoking but here there was no correlation between the incidence of compounds and duration of ICU stay nor was there any difference between the smokers and non-smokers. Less common were defects in microtubule number and arrangement. These types of abnormality are believed to originate during ciliogenesis, particularly during periods of rapid cell turnover such as that after infection. In the ICU population therefore it wouldn't have been surprising if they had been present in higher proportions. One would expect patients with respiratory infection to have a higher than normal amount of cilial abnormalities as this has been demonstrated previously. The total proportion of abnormal cilia however did not exceed 15% in any of

the patients and is unlikely to be of clinical significance. The majority of patients in this study had some kind of infection although none had more than 15% abnormal cilia. Several had pneumonia. We were unable to demonstrate any difference in the incidence of abnormalities between the patients, probably because of their diversity and the small sample size.

The fact that none of the patients had more than 15% abnormal cilia is somewhat hopeful because it would suggest that the mechanism of impaired mucus transport in these patients is not mediated in this way. However, there were only a small number of patients in this study and although no relationship between the duration of stay and incidence of cilial abnormalities was found, few of the samples came from patients who had been in the ICU longer than eight days. It has been noted before that it may take considerably longer for a significant number of cilial abnormalities to manifest. Longer stay patients therefore need to be studied in future.

Cilial abundance was highly variable among the patients, ranging from 0% to 97.3%. There was no correlation between duration of stay and abundance. Tracheal damage due to tracheal intubation and ventilation has been documented though there is a lack of research which quantitates cilial abundance in critically ill patients. The study by Konrad et al (1995) was limited by its utilisation of a semi-quantitative technique, classifying cilial abundance into three discrete categories: ciliated area greater than 75%, between 75 and 50%, and less than 50%. The other major limitation was that the duration of stay did not exceed three days. The present data show that cilial abundance can still be substantial even after one month in critical care. This finding is also encouraging for ICU patients because it indicates that after presumed damage due to intubation, recovery of the epithelium does

take place. More research needs to be done in order to determine why this should be the case for some patients and not others. Other factors that are known to have an effect include tube movements, suction and bronchfiberoscopy, inadequate humidification, high inspiratory oxygen concentration, and high ventilation pressure. Anaesthesia has also been implicated but work presented in this thesis shows no deleterious effect of two particular agents, midazolam and propofol, on cilial survival *in vitro*. Information that was known about the patients in this study included age, gender, duration of stay, $Fi0_2$, and smoking status but the sample size was not sufficient to explore the contribution of these factors individually. Another major drawback of this study is that the patients had several different underlying conditions and patients without airway disease were not excluded. The APACHE II score, was found to have a significant correlation with cilial abundance ($r= -0.65$, $p=0.02$), a finding which is of unknown importance, and suggests that more research is needed in this area.

In conclusion, it is encouraging to find that the ultrastructure and abundance of cilia need not necessarily be adversely affected by intensive care in all patients. It necessitates further research to confirm these findings in longer stay patients and to establish which factors contribute to reduced cilial abundance. This preliminary study has confirmed several previous findings and emphasised the need to count a large number of cilia. The patients in this group were diverse, and several had pre-existing respiratory conditions which could have affected their results. One of the initial aims of this study was to track the incidence of cilial abnormalities in individual patients from the beginning of their stay in intensive care until their discharge. In practice this did not happen, one reason, perhaps being because it was not possible to anticipate how long an individual would remain in intensive care. The preliminary nature of this study meant that this was not possible at this time. No

exclusion criteria were set during this phase of the study otherwise there would have been even fewer patients. Now confident that the samples obtained are of sufficient quality, the next phase of the study should consider limiting the study population to those who, at the time of admission, have a 'healthy' respiratory system. This may limit the patients to those who for example are admitted with a head injury.

CHAPTER SEVEN

General Discussion

Currently one in ten hospitalised patients succumbs to a hospital acquired infection (HAI) which represents a particularly serious clinical problem in the intensive care unit (Court and CS 1992). Pneumonia is the third most common infection after urinary tract and surgical wound infections and is a cause of considerable morbidity and mortality (Kelsey et al., 2000). Intensive care patients are the most susceptible group of patients to nosocomial pneumonia in whom mortality rates have been reported of up to 40% (Cooke and Watson 1996). Clinical and epidemiological studies have revealed many potential risk factors for developing an infection during intensive care, which include intubation, mechanical ventilation, anaesthesia, and cigarette smoking (Court and CS 1992; Vincent et al., 1995; Cooke and Watson 1996; Kelsey et al., 2000).

The major defence mechanism of the airways is the mucociliary transport system, which comprises the cilia and overlying mucus. Damage to this system is a likely mechanism through which certain factors may predispose to respiratory infection, for example, by promoting secretion retention and allowing bacterial colonisation. Abundance and ultrastructural integrity of the cilia are critical features of the mucociliary defence system and have not previously been extensively studied in critical illness. The aims of this programme of study, therefore, were to investigate the form and function of cilia in critically ill patients and to investigate known risk factors (specifically anaesthesia and cigarette smoking) independently. This novel approach has the potential to lead to improved treatment protocols for patients at particular risk.

This research has examined for the first time the mucociliary transport system in critically ill patients using novel and robust techniques. The amount of cilial ultrastructural abnormalities was highly variable among ICU patients. This was not known previously and undoubtedly necessitates further investigation owing to the importance of the cilia in optimal mucus clearance. It was thought previously that cilial abnormalities were induced by smoking and that they were irreversible. Chapter Five, however, showed that the increased risk of infection that smokers face in intensive care is not likely to be mediated through pre-existing cilial abnormalities since asymptomatic smokers had no more abnormal cilia than non-smokers. Of particular clinical relevance is the finding that neither midazolam nor propofol had an adverse effect on cilia survival. In addition, halothane was shown not only to have an adverse effect on CBF but also on amplitude of the beat, which is also important in mucus clearance. These findings, along with those of previous research, may influence the choice of agent used in intensive care patients.

The success of this research in testing the original hypotheses is outlined below:

1. The mucociliary transport system of critically ill patients declines with increasing length of stay in intensive care.

Chapter Six of this thesis reports a novel approach to studying abnormalities and abundance of cilia in critically ill patients. This approach utilised a modification of the method described in Chapter Three for quantification of cilial abundance, and counting larger numbers of cilia from several different sections for the determination of ultrastructural abnormalities. This study provided new information because samples were taken from patients beyond three days of mechanical ventilation. ICU patients were found to have variable amounts of cilial abnormalities, which necessitates further research. Several of the samples contained focal areas of what appeared to be single cells containing cross-sectioned cilia that had a combination of particular abnormalities. These were

frequently missing central tubules associated with loss of membrane integrity and partially degraded B- subunits. The appearance of the cilia in conjunction with consultation of the literature led to the conclusion that these particular cilia represented dead or dying cells in the process of autolysis. It is possible that these represented infected cells. This illustrated the major difficulty in assessing cilial abnormalities, and that it is imperative to count not just a large number of cilia but also to take several diverse sections. This was not done in previous research (Konrad et al., 1995). The quantification of ultrastructural abnormalities in Konrad's study involved counting up to 250 transverse cross sections of cilia. However, the focal nature of abnormalities necessitates the examination of larger numbers. In the present study, there was no correlation between the incidence of abnormal cilia and the length of ICU stay but the sample size was probably inadequate, particularly given that most the samples were taken within two weeks of admission. However, this was a preliminary study with the aim to elucidate the natural history (decline?) of the mucociliary system in intensive care. In practice only three of the patients had repeat samples taken and longer term studies are therefore needed. In addition to length of stay, information that was known about the patients included diagnosis, smoking history, inspired oxygen concentration, and microbiology but there were no associations between any of these variables and the incidence of abnormal cilia. Most of the patients were shown to have bacterial colonisation and even in the absence of overt infection, bacteria and their products have been shown to impair mucociliary clearance. Several bacteria have been shown to impair the mucociliary clearance system and damage the ciliated epithelium (Wilson et al., 1996). For instance, products of *Pseudomons aeruginosa*, pyocynin and 1-hydroxyphenazine, can impair ciliary function *in vivo* and have been shown to cause a decrease in CBF, associated with ciliary dyskinesia, *in vitro* (Wilson et al., 1987). Ultrastructural damage to cilia has also been associated with viral and bacterial infection. For example, focal areas of abnormal cilia have been demonstrated in the nasal epithelium of children during acute viral infection. The first week of infection was marked by a large

number of abnormal cilia with a gradual return to normal ultrastructure which was complete within ten weeks (Carson et al., 1985). Others may affect cilia survival. For example, human ciliated mucosa suffered 50% loss of cilia after 18 hours incubation with *Mycobacterium tuberculosis*, which was also associated with marked epithelial damage and internalisation within the epithelial cells (Feldman, Anderson et al 1998). Colonization is of significant importance in ICU patients and may result from several factors including inhalation, aspiration from the upper airways, or translocation from the upper airways by endotracheal tubes, suction catheters, and endoscopes. A difficulty concerning the measurement of cilial abundance in ICU patients is that even in healthy airways areas of up to $1mm^2$ can be devoid of cilia and small areas of squamous metaplasia may be considered normal. This point was not addressed in previous research and can only be overcome by taking repeat samples over an extended period of time. In previous research in intensive care patients cilial abundance was measured using a crude semi-quantitative technique which involved classifying the samples into one of three groups: greater than 70% cilial coverage, between 50 – 70%, and less than 50% coverage (Konrad et al., 1995). Presented in Chapter Three of this thesis is a robust objective technique to accurately quantify cilial abundance by scanning electron microscopy and semi-automated image analysis. Abundance was assessed by Ross Kay and is discussed further in his thesis. It was found to highly variable and there was once again no clear link with length of stay.

It was one of the initial aims to measure CBF in the ICU samples. Unfortunately this was not possible due to technical difficulties. Ideally, *in vivo* studies could also be carried out. Several methods by which to measure MTR have been described and this has already been measured in ICU patients, but only in the short term.

In summary therefore the original hypothesis that the mucociliary transport system of critically ill patients declines with increasing length of stay in intensive care, could not be

confirmed. Evidently there are many contributing factors which predispose patients to infection by damage to the mucociliary transport system. Among them but not limited to, they include microbial colonisation (described above) and pre-existing illness, which could not be addressed in this study due to its preliminary nature. However, the present study has shown that is *not* inevitable that the mucociliary transport system is *always* adversely affected by prolonged intensive care. This fact clearly necessitates more in depth research. However, this also means that at the present time there would appear to be no practical implications of this part of the present research for clinical care!

2. The intravenous anaesthetic agents midazolam and propofol have a deleterious effect upon cilia survival *in vitro*.
3. Halothane induces detrimental changes to cilia beat form in vitro, in addition to its effect upon cilia beat frequency.

Anaesthesia is another known risk factor for the development of nosocomial infection during intensive care. MTR during anaesthesia has been shown to be reduced on several occasions previously (Forbes 1976; Forbes and Gamsu 1979; Konrad et al., 1993). Furthermore, specific agents used to provide anaesthesia have been shown to cause a time and dose dependent decrease in CBF (Raphael et al., 1996; Raphael, et al. 1996). Abundance of cilia as well as CBF is of critical importance to mucociliary clearance and while the effects of anaesthesia on CBF are well established, little was previously known about any deleterious effect on cilial abundance. A rat model was chosen initially to provide a reproducible technique which could be used to perform specific experiments. Half rings of rat trachea were maintained in culture for a period of five days during which two anaesthetic drugs midazolam and propofol were tested in order to elucidate any effect on cilia survival. This was done using a highly quantitative technique which has not previously been described within this context. Raphael et al (1996) exposed human nasal turbinate explants to serial concentrations of midazolam and propofol and, using light microscopy and a crude scoring system (cilia either present or absent) found that there was

a time and dose dependent effect of midazolam on cilia survival. The work presented in Chapter Four represents a much improved novel approach, which incorporated the use of SEM and image analysis to quantify cilial abundance after exposure. There appeared to be an increased abundance of cilia in the half-rings exposed to midazolam when compared to the control, however, as discussed in Chapter Four, although statistically significant the effect is unlikely to be of any clinical significance. There was no difference in abundance after exposure to propofol. Thus, there was no deleterious affect of either anaesthetic in terms of cilial abundance, contradicting the original hypothesis. This was in contrast to suggestions from the previous work but also in contrast, this was a robust, objective study in which cilia survival was the primary outcome measure. These agents have previously been studied in experiments on CBF with no adverse effects observed. Together, these findings indicate that these agents can be used in confidence that they are not likely to exacerbate secretion retention unlike others such as halothane. There is little information regarding the effect of midazolam or propofol on MTR but any reduction in MTR associated with these agents would appear unlikely to be due to decreased CBF or loss of cilia. It is important to remember, however, that the mucociliary transport system in ICU patients is already under threat from factors other than the anaesthetic agent used and the impaired system may be more susceptible to damage. *In vitro* work is also limited due to absence of regulatory influences.

In January 1998 an opportunity arose to carry out a piece of collaborative research with a group of researchers in Finland over a period of three months. The aim was to utilise HRE cells and high speed digital video, a method which this group had previously published, for investigating the effect of other anaesthetic agents on the beat amplitude and synchrony as well as CBF. The adverse effect of halothane on MTR and CBF is already well-documented, which this study confirmed. In addition, not previously known was that the decrease in CBF was associated with a decrease in amplitude. There were significant

correlations between CBF, amplitude and synchrony which indicates that falls in CBF may also be associated with dyskinetic movement. The original hypothesis therefore proved to be correct.

The implications for clinical care of the intensive care patient in light of these studies are clearly limited by their *in vitro* nature. However, it may be advantageous to use those agents for which there is no evidence of an adverse effect on mucociliary structure or function in place of anaesthetic agents which have indisputably adverse effects, if otherwise clinically appropriate.

4. There is a higher incidence of cilial abnormalities in asymptomatic smokers than in healthy non-smokers.

The adverse effect of smoking on MTR and CBF has been demonstrated previously and smoking is one known risk factor for developing a respiratory infection during intensive care. The role of cilial ultrastructural abnormalities in predisposing smokers to nosocomial infection is unknown however and there is conflicting evidence regarding the incidence of cilial abnormalities in smokers prior to infection. Asymptomatic smokers are known to suffer a loss of cilia and mucus hypersecretion, resulting in an increased risk of impaired mucociliary transport. This is thought to be mediated in part by elastase, released by neutrophils during the inflammatory response to cigarette smoke. Research has suggested that neutrophils may also have a role in the formation of abnormal cilia in smokers. Verra et al (1995) found a higher percentage of abnormal cilia in smokers and ex-smokers compared to non-smokers and controls which suggested that these changes were permanent. Their study was limited by small patient number in the control group and by the fact that the samples did not appear to be subject to a blinding process before examination.

The study presented in Chapter Five addressed the above hypothesis and consisted of a much larger group of asymptomatic patients than any other previous research from whom samples of tracheal cilia were examined according to a robust and rigorously blinded protocol. This study was unique in its aim to study only asymptomatic patients in order to determine whether or not there are an increased number of cilial abnormalities in smokers prior to the onset of disease. In contrast to previous research this study could not detect any difference in the proportion of abnormal cilia between asymptomatic smokers and non-smokers (it was sufficiently powered to detect a difference of as little as 2% between the two groups). The original hypothesis was therefore not proven. Symptomatic smokers (i.e. those with CSP, bronchitis etc) have previously been found to have an increased proportion of cilial abnormalities. Together, these findings indicate that loss of cilia, decreased CBF and/ or mucus hypersecretion may be more important as factors which predispose smokers to infection and that the increased incidence of abnormal cilia thereafter are secondary to infection. However, there were few heavy smokers in the study and as such the implications of this research are limited Further knowledge of the aetiology of cilial abnormalities may be elucidated from a longitudinal study of asymptomatic smokers and non-smokers over a period of years. In addition, the present study found a significant correlation between age and proportion of peripheral cilial abnormalities only in the smokers. It would therefore be useful to include in a future study a greater number of heavier asymptomatic smokers. At present it is not possible to confirm that cilial abnormalities in asymptomatic smokers do not contribute to the development of nosocomial infection in intensive care patients.

Further work

There is no doubt as to the importance of the ultrastructure and function of cilia for efficient mucociliary transport. However, it is dependent not only upon the cilia, but also upon the amount and rheological properties of mucus and the interaction between the cilia and mucus. If any of these are impaired, mucociliary transport is inevitably adversely affected. Another approach to studying the mucociliary transport system in the critically ill would be to include studies on the properties of mucus. Several parameters relating to the viscoelastic properties of mucus are possible to study. Transportability of mucus samples is commonly measured using the mucus-depleted frog palate and surface mechanical impedence by the rolling ball technique (Rubin 1998). These techniques have been used to study the sputum from patients with cystic fibrosis, asthma, and chronic bronchitis and could be applied to the study of sputum from ICU patients.

Other suggestions for further research in order to improve choice of clinical care:

- Study longer term ICU patients – longitudinal studies may be more useful to identify patients at particular risk. Include serial measures of:
    - cilial abnormalities/ abundance
    - *in vivo* mucociliary activity
    - amount and viscoelastic properties of mucus

- Asses risk of any new anaesthetic agent or combinations of agents which may affect mucociliary transport – short term and long term effects on various aspects of cilial abnormalities, abundance and function.

- Longitudinal study of smokers – identify smokers at particular risk of developing infection.

At present, control of the mucociliary transport system is not fully understood and as it becomes elucidated, potential mechanisms for risk factors will be revealed. A greater understanding of the contribution of independent risk factors as well as their interactions has the potential benefit of leading to the development of novel therapies and improved treatment protocols.

Afzelius BA (1959). Electron microscopy of the sperm tail; results obtained with a new fixative. *Journal of Biophysical Biochemical Cytology* **5: 269-278**

Afzelius BA (1976). A human syndrome caused by immotile cilia. *Science* **193 (4250): 317-9**

Afzelius BA (1998). Genetics and pulmonary medicine. 6. Immotile cilia syndrome: past, present, and prospects for the future. *Thorax* **53 (10): 894-7**

Afzelius BA (1999). Asymmetry of cilia and of mice and men. *International Journal of Developmental Biology* **43 (4): 283-6**

Afzelius BA, Gargani G, Romano C (1985). Abnormal length of cilia as a possible cause of defective mucociliary clearance. *European Journal of Respiratory Diseases* **66 (3): 173-80**

Agius AM, Smallman LA, Pahor AL (1998). Age, smoking and nasal ciliary beat frequency. *Clinical Otolaryngology* **23 (3): 227-30**

Albert RE, Lippmann M, Briscoe W (1969). The characteristics of bronchial clearance in humans and the effects of cigarette smoking. *Archives of Environmental Health* **18 (5): 738-55**

Albert RE, Berger J, Sanborn K, Lippmann M (1974). Effects of cigarette smoke components on bronchial clearance in the donkey. *Archives of Environmental Health* **29 (2): 96-101**

Alexander I, Ritchie BC, Maloney JE, Hunter CR (1975). Epithelial surfaces of the trachea and principal bronchi of the rat. *Thorax* **30: 171-177**

Asmundsson T, Kilburn K (1970). Mucoiliary clearance rates at various levels in dog lungs. *American Review of Respiratory Disease* **102: 388 - 397**

Auerbach O, Hammond EC, Garfinkel L (1979). Changes in bronchial epithelium in relation to cigarette smoking. *New England Journal of Medicine* **300 (8): 381-5**

Ayers MM, Jeffery PK (1988). Proliferation and Differentiation in Mammalian Airway Epithelium. *European Respiratory Journal* **1 (1)**: **58-80**

Barnes SD, Agee CC, Peace RJ, Leffler CW (1983). Effects of elevated $P0_2$ upon tracheal explants. *Respiration Physiology* **53 (3)**: **285-93**

Battista SP, Denine EP, Kensler CJ (1972). Restoration of tracheal mucosa and ciliary particle transport activity after mechanical denudation in the chicken. *Toxicology and Applied Pharmacology* **22: 59 - 69**

Beckers S, Camu F (1991). The anesthetic risk of tobacco smoking. *Acta Anaesthesiologica Belgica* **42 (1)**: **45-56**

Boat FT (1979). Studies of oxygen toxicity in cultured human neonatal respiratory epithelium. *The Journal of Pediatrics* **95 (5, pt2)**: **916-919**

Camner P, Philipson K (1972). Tracheobronchial clearance in smoking-discordant twins. *Archives of Environmental Health* **25 (1)**: **60-3**

Carson JL, Collier AM, Hu S, Smith CA, Stewart P (1987). The appearance of compound cilia in the nasal mucosa of normal human subjects following acute, in vivo exposure to sulfur dioxide. *Environmental Research* **42 (1)**: **155-65.**

Carson JL, Collier AM, Fernald GW, Hu SC (1994). Microtubular discontinuities as acquired ciliary defects in airway epithelium of patients with chronic respiratory diseases. *Ultrastructural Pathology* **18 (3)**: **327-32**

Carson JL, Collier AM, Hu SS (1985). Acquired ciliary defects in nasal epithelium of children with acute viral upper respiratory infections. *New England Journal of Medicine* **312 (8)**: **463-8**

Cavaliere F, Schiavello R, Masieri S, Passali D (1983). Mucociliary flow in the nose during general and epidural anesthesia. *Acta Anaesthesiologica Belgica* **34 (1)**: **33-9.**

Centanni S, Camporesi G, Tarsia P, Guarnieri R, Allegra L (1998). Effect of atropine on ciliary beat in human upper respiratory tract epithelial cells. *International Journal of Tissue Reactions* **20 (4): 131-6.**

Cervin A, Lindberg S, Mercke U (1995). Effects of halothane on mucociliary activity in vivo. *Otolaryngology - Head and Neck Surgery* **112 (6): 714-22**

Chalon J, Loew DA, Malebranche J (1972). Effects of dry anesthetic gases on tracheobronchial ciliated epithelium. *Anesthesiology* **37 (3): 338-43**

Chalon J, Tayyab MA, Ramanathan S (1975). Cytology of respiratory epithelium as a predictor of respiratory complications after operation. *Chest* **67 (1): 32-35**

Chandra T, Yeates DB, Miller IF, Wong LB (1994). Stationary and nonstationary correlation-frequency analysis of heterodyne mode laser light scattering: magnitude and periodicity of canine tracheal ciliary beat frequency in vivo. *Biophysical Journal* **66 (3 Pt 1): 878-890**

Chang L-Y, Wu R, Nettesheim P (1985). Morphological changes in rat tracheal cells during the adaptive and early growth phase in primary cell culture. *Journal of Cell Science* **74: 283-301**

Chilvers MA, O'Callaghan C (2000). Analysis of ciliary beat pattern and beat frequency using digital high speed imaging: comparison with the photomultiplier and photodiode methods. *Thorax* **55 (4): 314-7**

Chopra S (1978). Effect of atropine on mucociliary transport velocity in anaesthetized dogs. *American Review of Respiratory Disease* **118: 367 - 371**

Clark AB, Randell SH, Nettesheim P, Gray TE, Bagnell B, Ostrowski LE (1995). Regulation of ciliated cell differentiation in cultures of rat tracheal epithelial cells. *American Journal of Respiratory Cell and Molecular Biology* **12: 329-338**

Clary Meinesz CF, Cosson J, Huitorel P, Blaive B (1992). Temperature effect on the ciliary beat frequency of human nasal and tracheal ciliated cells. *Biology of the Cell* **76 (3): 335-8**

Coggins CR, Fouillet XL, Lam R, Morgan KT (1980). Cigarette smoke induced pathology of the rat respiratory tract: a comparison of the effects of the particulate and vapour phases. *Toxicology* **16 (2): 83-101**

Cooke RPD, Watson NA (1996). Pneumonia in the ICU. Different approaches to management. *British Journal of Intensive Care* **April : 126-133**

Court CA, CS G (1992). Nosocomial pneumonia in the intensive care unit: mechanisms and significance. *Thorax* **47: 465 - 473**

Dalhamn (1962). Frequency of ciliary beat measured with a photo-sensitive cell. *Nature* **196 : 592-593**

Dalhamn T (1966). Effect of cigarette smoke on ciliary activity. *American Review of Respiratory Disease* **93 (3): Suppl: 108-14**

Dalhamn T (1970). In vivo and in vitro ciliotoxic effects of tobacco smoke. *Archives of Environmental Health* **21 (5): 633-4**

Davies P, Kistler GS (1975). The assessment of tobacco smoke toxicity in organ culture. II. Ultrastructural studies on the immediate response of foetal rabbit tracheal epithelium to short-term exposures of whole smoke. *Experientia* **31 (6): 682-4**

De Iongh R, Rutland J (1989). Orientation of respiratory tract cilia in patients with primary ciliary dyskinesia, bronchiectasis, and in normal subjects. *Journal of Clinical Pathology* **42 (6): 613-9**

De Iongh R, Rutland J (1995). Ciliary defects in healthy subjects, bronchiectasis, and primary cliary dyski.nesia. *Ameriacn Journal of Respiratory and Critical Care Medicine* **151 : 1559 - 1567**

Dellinger OP (1909). The cilium as a key to the structure of contractile protoplasm. *Journal of Morphology* **20: 171-210**

Devalia JL, Sapsford RJ, Rusznak C, Toumbis MJ, Davies RJ (1992). The effects of salmeterol and salbutamol on ciliary beat frequency of cultured human bronchial epithelial cells, in vitro. *Pulmonary Pharmacology* **5 (4): 257-63.**

DiBenedetto G, Manara-Shediac F, mehta A (1991). Effect of cyclic AMP on ciliary activity of human respiratory epithelium. *European Respiratory Journal* **4: 789 - 795**

Dilworth J, White R (1992). Postoperative chest infection after upper abdominal surgery: an important problem for smokers. *Respiratory Medicine* **86 (3): 205-210**

Dobell C: "Antony van Leeuwenhoek and his little animals". New York, Russell and Russell, 1958

Duchateau GSMJE, Graamans K, Zuidema J, Merkus FWHM (1985). Correlation between nasal ciliary beat frequency and mucus transport rate in volunteers. *Laryngoscope* **95 : 854-859**

Ebert RV, Terracio MJ (1975). The bronchiolar epithelium in cigarette smokers. Observations with the scanning electron microscope. *American Review of Respiratory Disease* **111 (1): 4-11**

Fawcett D, Porter KR (1954). A study of the fine structure of ciliated epithelia. *Journal of Morphology* **94: 221-281**

Fonzi L, Lungarella G, Santi MMD (1982). Ultrastructural observations on morphogenesis of atypical cilia. *Anat Anz Jena* **151: 151-159**

Forbes A (1976). Halothane depresses mucociliary flow in the trachea. *Anesthesiology* **45: 59-63**

Forbes AR, Gamsu G (1979). Lung mucociliary clearance after anesthesia with spontaneous and controlled ventilation. *American Review of Respiratory Disease* **120 (4): 857-862.**

Forbes AR, Horrigan RW (1977). Mucociliary flow in the trachea during anesthesia with enflurane, ether, nitrous oxide, and morphine. *Anesthesiology* **46 (5): 319-21.**

Fox B, Bull TB, Oliver TN (1983). The distribution and assessment of electron-microscopic abnormalities of human cilia. *European Journal of Respiratory Diseases. Supplement* **127: 11-8**

Friedmann I, Bird ES (1971). Ciliary structure and, ciliogenesis, microvilli. *Laryngoscope* **81: 1852 - 1868**

Gamsu G, Singer MM, Vincent HH, Berry S, Nadel JA (1979). Postoperative impairment of mucous transport in the lung. *American Review of Respiratory Disease.* **114: 673-679**

Gatto LA (1993). Cholinergic and adrenergic stimulation of mucociliary transport in the rat trachea. *Respiration Physiology* **92** : **209 - 217**

George DL (1995). Epidemiology of nosocomial pneumonia in intensive care unit patients. *Clinics in Chest Medicine* **16 (1): 29 - 43**

Gibbons IR, Grimstone AV (1960). On flagellar structure in certain flagellates. *Journal of Biophysical and Biochemical Cytology* **7: 697-716**

Goodman RM, Yergin BM, Landa JF, Golivanux MH, Sackner MA (1978). Relationship of smoking history and pulmonary function tests to tracheal mucous velocity in nonsmokers, young smokers, ex-smokers, and patients with chronic bronchitis. *American Review of Respiratory Disease* **117 (2): 205-14**

Gordon RE, Williams KB, Puszkin S (1982). Immune localisation of calmodulin in the ciliated cells of hamster tracheal epithelium. *The Journal of Cell Biology* **95: 57 - 63**

Grant RE (1835). On the nervous system of Beroe pileus, Lam., and on the structure of its cilia. *Trans. Zool. Soc. Lond.* **1** : **9-12**

Gross P, Antwerpen C (1983). Nosocomial infections and hospital deaths. *The American Journal of Medicine* **75: 658 - 662**

Gryczynska D, Kobos J, Zakrzewska A (1999). Relationship between passive smoking, recurrent respiratory tract infections and otitis media in children. *International Journal of Pediatric Otorhinolaryngology* **49 Suppl 1: S275-S278**

Gyi A, O'Callaghan C, Langton JA (1994). Effect of halothane on cilia beat frequency of ciliated human respiratory epithelium in vitro. *British Journal of Anaesthesia* **73 (4)**: **507-10**

Hagiwara H, Ohwada N, Aoki T, Takata K (2000). Ciliogenesis and ciliary abnormalities. *Medical Electron Microscopy* **33: 109-114**

Halsey MJ: Potency, physical properties and molecular mechanisms of inhalational anaesthetics., International Practice of Anaesthesia. Edited by Jr. CP-RaBRB. Oxford, Butterworth-Heinemann, 1996, pp Chapter 10

Hamasaki T, Murtaugh TJ, Satir BH, Satir P (1989). In vitro phosphorylation of paramecium axonemes and permeabilized cells. *Cell Motility and the Cytoskeleton* **12**: **1 - 11**

Hamasaki T, Barkalow K, Richmond J, Satir P (1991). cAMP stimulated phosphorylation of an axonemal polypeptide that copurifies with the 22S dynein arm regulates microtubule translocation velocity and swimming speed in paramecium. *Proceedings of the National Academy of Sciences of the USA* **88**: **7918 - 7922**

Hann HC, Hall AP, Raphael JH, Langton JA (1998). An investigation into the effects of midazolam and propofol on human respiratory cilia beat frequency in vitro. *Intensive Care Medicine* **24 (8)**: **791-4**

Hasani A, Spiteri MA, Pavia D, Lopez-Vidriero MT, Agnew JE, Clarke SW (1992). Effect of temazepam on tracheobronchial mucus clearance. *Thorax* **47 (4)**: **298-300**

Hilding AC (1964). Time-lapse relation to changes in the respiratory epithelium after minimal trauma. *Acta Otolaryngology* **57: 352**

Hilding DA, Hilding AC (1966). Ultrastructure of tracheal cilia and cells during regeneration. *Annals of Otology, Rhinology, and Laryngology* **75: 281 - 296**

Ho JC, Chan KN, Hu WH, Lam WK, Zheng L, Tipoe GL, Sun J, Leung R, Tsang KW (2001). The Effect of Aging on Nasal Mucociliary Clearance, Beat Frequency, and Ultrastructure of Respiratory Cilia. *American Journal of Respiratory and Critical Care Medicine* **163: 983-988**

Horan T, White J, Jarvis W, al e (1986). Nosocomial infection suveillance. *MMWR* **35 (1SS): 17SS - 29SS**

Huberman D (1993). A device for measuring mucociliary activity in the human bronchi during fibre-optic bronchoscopy. *Acta Otolaryngology* **113 (5): 683-6**

Houtmeyers E, Gosselink R, GayanRamirez G, Decramer M (1999). Regulation of mucociliary clearance in health and disease. *European Respiratory Journal* **13 (5): 1177-1188**

Hybbinette JC (1982). A pharmacological evaluation of the short-term effect of cigarette smoke on mucociliary activity. *Acta Otolaryngologica* **94 (3-4): 351-9**

Hybbinette JC, Mercke U (1982). Effects of the parasympathomimetic drug metacholine and its antagonist atropine on mucociliary activity. *Acta Otolaryngologica* **93: 465 - 473**

Iravani J (1972). Ciliary activity in the respiratory tract following chronic exposure to cigarette smoke. *Rehabilitation; Sozialmedizin Physikalischemedizin Praventivmedizin* **25 (1): 40-2**

Irlbeck D (1998). Normal mechanisms of heat and moisture exchange in the respiratory tract. *Respir Care Clinics of North America* **4 (2): 189-98**

Irwin R, Erickson A, MR p (1982). Prediction of tracheobronchial colonisation in curretn cigarette smokers with chronic obstructive bronchitis. *The Journal of Infectious Diseases* **145: 234-241**

IuN K, Ivanov AI, NIu V, Chernekhovskaia NE, Faradzheva NA (1991). The state of bronchial ciliated epithelium and mucociliary transport during chronic bronchitis in long-term smokers. *Klin Med (Mosk)* **69 (5): 50-53**

Jeffery PK, Reid L (1975). New observations on rat airway epithelium: a quantitative and electron microscopic study. *Journal of Anatomy* **120 (2): 295-320**

Joki S, Toskala E, Saano V, Nuutinen J (1998). Correlation between ciliary beat frequency and the structure of ciliated epithelia in pathologic human nasal mucosa. *Laryngoscope* **108 (3): 426-30**

Jones R, Reid L (1979). ß-agonists and rat airway secretory cell hyperplasia. *Federation Proceedings* **38: 1155**

Kaartinen L, Nettesheim P, Adler KB, Randell SH (1993). Rat tracheal epithelial cell differentiation in vitro. *In Vitro Cell Deveopment Biology.* **29A: 481-492**

Kai H, Yamamoto S, Takahama K, Miyata T (1990). Influence of corticosterone on tracheal mucociliary transport in pigeons. *Japanese Journal of Pharmacology* **52 (3): 496-9.**

Kaminski EJ, Fancher OE, Calandra JC (1968). In vivo studies of the ciliastatic effects of tobacco smoke. Absorption of ciliastatic components by wet surfaces. *Archives of Environmental Health* **16 (2): 188-93**

Kawada H, Kudo Y, Takizawa T (1991). Cigarette smoke and bronchoepithelium. *nihon kyobu shikkan gakkai zasshi* **29 (2): 197-201**

Kelsey MC, Mitchell CA, Griffin M, Spencer RC, Emmerson AM (2000). Prevalence of lower respiratory tract infections in hospitalised patients in the United Kingdom and Eire - results from the second national prevalence survey. *Journal of Hospital Infection* **46: 12 - 22**

Kensler CJ, Dalhamn T, Rylander R (1967). Cigarette smoke and ciliastasis. *Archives of Environmental Health* **14 (2): 371-3**

Kobayashi K, Salathe M, Pratt M, al e (1992). Mechanism of hydrogen peroxide induced inhibition of sheep airway cilia. *American Journal of Respiratory Cell and Molecular Biology* **6: 667 - 673**

Konrad F, Schreiber T, Grunert A, Clausen M, Ahnefeld FW (1992). Measurement of Mucociliary Transport Velocity in Ventilated Patients - Short-Term Effect of General-Anesthesia On Mucociliary Transport. *Chest* **102 (5): 1377-1383**

Konrad FX, Schreiber T, Brechtkraus D, Georgieff M (1993). Bronchial Mucus Transport in Chronic Smokers and Nonsmokers During General-Anesthesia. *Journal of Clinical Anesthesia* **5 (5): 375-380**

Konrad F, Schreiber T, Hahnel J, Kilian J, Georgieff M (1994). Effect of Theophylline On Mucociliary Transport in Ventilated Intensive-Care Patients. *Anaesthesist* **43 (2): 101-106**

Konrad F, Schreiber T, Brechtkraus D, Georgieff M (1994). Mucociliary Transport in ICU Patients. *Chest* **105 (1): 237-241**

Konrad F (1995). Clinical aspects of mucociliary transport in anesthesia and intensive-care medicine. *Acp-Applied Cardiopulmonary Pathophysiology* **5 (4): 249-255**

Konrad F, Schiener R, Marx T, Georgieff M (1995). Ultrastructure and Mucociliary Transport of Bronchial Respiratory Epithelium in Intubated Patients. *Intensive Care Medicine* **21 (6): 482-489**

Konrad F, Mezody M, Goertz A, Marx T, Georgieff M (1996). The effect of a heat and moisture exchanger (HME) on bronchial mucus transport in a closed inhalation anaesthesia system. *Anaesthetist* **45 (9): 802-806**

Konrad F, Marx T, Schraag M, Kilian J (1997). Combination anesthesia and bronchial tranport velocity. Effects of anesthesia with isoflurane, fentanyl, vecuronium and oxygen-nitrous oxide breathing on bronchial mucus transport. *Anaesthesist* **46 (5): 403-7**

Konrad F, Schraag S, Marx T, Kilian J, Goertz A (1998). The effect of total intravenous anesthesia with propofol, alfentanil and vecuronium (TIVA) on bronchial mucosal transport. *Anasthesiol Intensivmed Notfallmed Schmerzther* **33 (3): 171-6.**

Konradova V, Janota J, Sulova J, Sukova B, Copova M (1987). The effect of short-term inhalation of humidified oxygen on the ultrastructure of respiratory tract epithelium. *Ceskoslovenska Pediatrie* **42 (11): 658-60**

Konradova V, Janota J, Sulova J, Sukova B, Copova M (1988). Effects of 90% oxygen exposure on the ultrastructure of the tracheal epithelium in rabbits. *Respiration* **54 (1): 24-32**

Laitinen A (1985). Ultrastructural organisation of intraepithelial nerves in the human airway tract. *Thorax* **40: 488 - 492**

Landa J, Hiesch J, Lebeaux M (1975). Effects of topical and general anaesthetic agents on tracheal mucus velocity of sheep. *Journal of Applied Physiology* **38: 946 - 948**

Lane BP, Miller SL (1976). Preparation of large numbers of uniform tracheal organ cultures for long term studies. I. Effects of serum on establishment in culture. *In Vitro* **12 (2): 147-154**

Langer M, Moscone P, Cigada M, al e (1989). Long term respiratory support and risk of pneumonia in critically ill patients. *American Review of Respiratory Disease* **118: 493 - 496**

Laurenzi GA, Yin S, Guarneri JJ (1968). Adverse effect of oxygen on tracheal mucus flow. *New England Journal of Medicine* **279 (7): 333-9**

Lee K, Park S (1980). Effect of halothane, enflurane, and nitrous oxide on tracheal ciliary activity in vitro. *Anesthesia and Analgesia* **59: 426-430**

Lee WI, Verdugo P (1976). Laser light scattering spectroscopy: a new application in the study of ciliary activity. *Biophysical Journal* **16 (9): 1115-1119**

Lemos M, Lichtenfels AJFC, E. Amaro J, Macchione M, Martins MA, King M, Bohm GM, Saldiva PHN (1994). Quantitative pathology of nasal passages in rats exposed to urban levels of air pollution. *Environmental Research* **66 (1): 87-95**

Levine S, Niederman M (1991). The impact of tracheal intubation on host defences and risks for nosocomial pneumonia. *Clinics in Chest Medicine* **12: 523 - 543**

Lungarella G, Fonzi L, Ermini G (1983). Abnormalities of Bronchial cilia in Patients with Chronic Bronchitis. *Lung* **161: 147-156**

Malick LE, Wilson RB (1975). Modified thiocarbohydrazide procedure for scanning electron microscopy: routine use for normal, pathological, or experimental tissues. *Stain Technology* **50 (4): 265-269**

Malinconico S, McCarl R (1982). Effect of halothane on cardiac sarcoplasmic reticulum Ca++ - ATPase at low Ca++ concentrations. *Molecular Pharmacology* **22 (1): 8-10**

Manawadu B, Mostow S, FM L (1979). Imapairment of tracheal ring ciliary activity by halothane. *Anesthesia and Analgesia* **58: 500-504**

Manton I (1952). The fine structure of plant cilia. *Symposia of the Society for Experimental Biology* **6: 306-319** University Press, Cambridge.

Marcy TW, Merrill WW (1987). Cigarette smoking and respiratory tract infection. *Clinics in Chest Medicine* **8 (3): 381-391**

Marfatia S, Donahoe PK, Hendren WH (1975). Effect of dry and humidified gases on the respiratory epithelium in rabbits. *Journal of Pediatric Surgery* **10 (5): 583-92**

McAuley JR, Anand VK (1998). Clinical significance of compound cilia. *Otolaryngology - Head and Neck Surgery* **118 (5): 685-687**

Meers P, Ayliffe G, Emerson A, al e (1981). Report on the National Survey of Infection in Hospitals. *Journal of Hospital Infection* **2: 1 - 51**

Mehta RM, Niederman MS (2003). Nosocomial Pneumonia in the Intensive Care Unit: Controversies and Dilemmas. *Journal of Intensive Care Medicine* **18 (4): 175-188**

Min YG, Kim IT, Park SH (1994). Mucociliary activity and ultrastructural abnormalities of regenerated sinus mucosa in rabbits. *Laryngoscope* **104 (12): 1482 - 1486**

Montesano R, Orci L (1985). Tumor-promoting phorbol esters induce angiogenesis in vitro. *Cell* **42 (2): 469-477**

Mortensen J, Lange P, Nyboe J, Groth S (1994). Lung mucociliary clearance. *European Journal Of Nuclear Medicine* **21 (9): 953-61**

Mossman BT, Craighead JE (1975). Long term maintenance of differentiated respiratory epithelium in organ culture I. Medium composition. *Proceedings of the Society for Experimental Biology and Medicine* **149: 227-233**

Muller OF (1786). "Animalcula Infusoria Fluviatilia et Marina.". *Havniae.* (In Satir, 1995)

Nikula KJ, Sabourin PJ, Frietag BC, Birdwhistell AJ, Hotchkiss JA, Harkema JR (1991). Biochemical and morphologic responses of rat nasal epithelia to hyperoxia. *Fundamental and Applied Toxicology* **17 (4): 675-83**

Nunn J, Sturrock J, Wills E, Richmond J, McPherson C (1974). The effect of inhlational anaesthetics on the swimming velocity of tetrahymena pyriformis. *Journal of Cell Science* **15: 537 - 554**

Obara H, Sekimoto M, Iwai S (1979). Alterations to the bronchial and bronchiolar surfaces of adult mice after exposure to high concentrations of oxygen. *Thorax* **34 (4): 479-85**

O'Donnell TV, Crocker TT, Nunes LL (1973). Maintenance of normal, metaplastic, and dysplastic states of adult human bronchial mucosa in organ culture. *Cancer Research* **33: 78-87**

Ostrowski LE, Randell SH, Clark AB, Gray TE, Nettesheim P (1995). Ciliogenesis of rat tracheal epithelial cells in vitro. *Methods in Cell Biology* **47: 57-63**

Paltieli Y, Fradis M, Ben-David J, Podoshin L, Shiti H, Kam Z (1997). In vivo measurement of human nasal mucociliary motility using a laser light scattering instrument. *Annals of Otology, Rhinology and Laryngology* **106 (10 Pt 1)**: **859-62**

Pavelka M (1976). Organ culture of adult rat and mouse epithelium: I. Ultrastructure following various culture periods. *Cell and Tissue Research* **165 (3)**: **371-382**

Pavia D, Thomson ML, Pocock SJ (1971). Evidence for temporary slowing of mucociliary clearance in the lung caused by tobacco smoking. *Nature* **231 (5301)**: **325-6**

Pearce AC, Jones RM (1984). Smoking and anesthesia: preoperative abstinence and perioperative morbidity. *Anesthesiology* **61 (5)**: **576-84**

Petros A, Bogle R, Pearson J (1993). Propofol stimulates nitric oxide release from cultured porcine endothelial cells. *British Journal of Pharmacology* **109**: **6 -7**

Pizov R, Takahashi M, Hirshman CA, Croxton T (1992). Halothane inhibition of ion transport of the tracheal epithelium. A possible mechanism for anesthetic-induced impairment of mucociliary clearance. *Anesthesiology* **76 (6)**: **985-9**

Plopper CG, Nishio SJ, Alley JL, Kass P, Hyde DM (1992). The role of the nonciliated bronchiolar epithelial (Clara) cell as the progenitor cell during bronchiolar epithelial differentiation in the perinatal rabbit lung. *American Journal of Respiratory Cell and Molecular Biology* **7: 603 - 613**

Plowman R, Graves N, Griffin M, Roberts JA, Swan AV, Cookson BD, Taylor L: Socio-economic burden of HAI. London, Central Public Health Laboratory Service London School of Hygiene and Tropical Medicine, 2000

Popp JA, Martin JT (1984). Surface topography and distribution of cell types in the rat nasal respiratory epithelium: scanning electron microscopic observations. *The American Journal of Anatomy* **169 (4)**: **425-436**

Puchelle E, Zahm J, Bertrand A (1979). Influence of age on bronchial mucociliary transport. *Scand J Respir Dis* **60: 307 - 313**

Purkinji JE, Valentin G (1835). De phaenomeno generli et fundamentali motus vibratorii continui in membranis cum externis tum internis animalium plurimorum et superiorum et inferiorum ordinum obvii. *Commentatio Physiologica*

Randall JT, Warr JR, Hopkins JM, McVittie A (1964). A single-gene mutation of *Chlamydomonas reinhardii* affecting motility: a genetic and electron microscope study. *Nature* **203: 912-914**

Raphael JH (1996): Effects of anaesthetic and sedative agents on human respiratory cilia in vitro., MD Dissertation. Department of Anaesthesia. Leicester.

Raphael JH, Strupish J, Selwyn DA, Hann HC, Langton JA (1996). Recovery of respiratory ciliary function after depression by inhalation anaesthetic agents: an in vitro study using nasal turbinate explants. *British Journal of Anaesthesia* **76 (6)**: **854-9**

Raphael JH, Selwyn DA, Mottram SD, Langton JA, O'Callaghan C (1996). Effects of 3 MAC of halothane, enflurane and isoflurane on cilia beat frequency of human nasal epithelium in vitro. *British Journal of Anaesthesia* **76 (1)**: **116-21**

Raphael JH, Butt MW (1997). Comparison of isoflurane with propofol on respiratory cilia. *British Journal of Anaesthesia* **79 (4)**: **473-5**

Rautiainen M, Collan Y, Nuutinen J (1986). Ciliary orientation - reproducible measurement from electron micrographs of respiratory cilia. *Journal of Ultrastructure and Molecular Structure Research.* **94 (3)**: **293**

Rautiainen M, Collan Y, Nuutinen J (1986). A method for measuring the orientation (beat direction) of respiratory cilia. *Archives Of Oto-Rhino-Laryngology* **243 (4)**: **265-8**

Rautiainen M, Collan Y, Karja J, Nuutinen J (1987). Artifacts in ultrastructure of respiratory cilia caused by various fixation procedures and different types of handling. *Journal of Otorhinolaryngology and related specialities.* **49: 193-198**

Rautiainen M, Collan Y, Nuutinen J, Afzelius BA (1990). Ciliary orientation in the immotile cilia syndrome. *European Archives Of Oto-Rhino-Laryngology* **247 (2)**: **100-3**

Rautiainen ME (1988). Orientation of human respiratory cilia. *European Respiratory Journal* **1 (3)**: **257-61**

Rautiainen M, Nuutinen J, Collan Y (1991). Short nasal respiratory cilia and impaired mucociliary function. *European Archives Of Oto-Rhino-Laryngology* **248 (5)**: **271-4**

Rautiainen M, Matsune S, Shima S, Sakamoto K, Hanamore Y, Ohyama M (1992). Ciliary beat of cultured human respiratory cells studied with differential interference microscope and high-speed video system. *Acta-Oto-Laryngolica* **112 (5)**: **845-851**

Rautiainen M, Collan Y, Nuutinen J (1992). Ciliary orientation - reproducible measurement from electron micrographs of respiratory cilia. *Journal of Ultrastructure and Molecular Structure Research* **94 (3)**: **293**

Rautiainen M, Matsune S, Yoshitsugu M, Ohyama M (1993). Degeneration of human respiratory cell ciliary beat in monolayer cell cultures. *European Archives of Oto-Rhino-Laryngology* **250 (2)**: **97-100**

Rautiainen M, Yoshitsugu M, Matsune S, Nuutinen N, Happonen P, Ohyama M (1994). Effect of Exogenous ATP and Physical Stimulation on Ciliary function Impaired by Bacterial Endotoxin. *Acta Otolaryngologica (Stockh)* **114: 337-340**

Rayner C, Rutman A, Dewar A, Greenstone M, Cole P, Wilson R (1996). Ciliary disorientation alone as a cause of primary ciliary dyskenesia syndrome. *American Journal of Respiratory and Critical Care Medicine* **153 (3)**: **1123-9**

Rayner CF, Rutman A, Dewar A, Cole PJ, Wilson R (1995). Ciliary disorientation in patients with chronic upper respiratory tract inflammation. *American Journal Of Respiratory And Critical Care Medicine* **151 (3 Pt 1)**: **800-4**

Riise GC, Larsson S, Andersson BA (1992). A bronchoscopic brush biopsy study of large airway mucosal pathology in smokers with chronic bronchitis and in healthy nonsmokers. *European Respiratory Journal* **5 (4)**: **382-386**

Rossman CM, Lee RM, Forrest JB, Newhouse MT (1983). Nasal cilia in normal man, primary ciliary dyskinesia and other respiratory diseases: analysis of motility and ultrastructure. *European Journal of Respiratory Diseases. Supplement* **127** : **64-70**

Rossman CM, Lee RM, Forrest JB, Newhouse MT (1984). Nasal ciliary ultrastructure and function in patients with primary ciliary dyskinesia compared with that in normal subjects and in subjects with various respiratory diseases. *American Review of Respiratory Disease* **129 (1)**: **161-7**

Roth Y, Baum GL, Aharonson EF, Priel Z, Teichtahl H, Modan M (1991). Human Invitro Nasal and Tracheal Ciliary Beat Frequencies - Comparison of Sampling Sites, Combined Effect of Medication, and Demographic Relationships. *Annals of Otology Rhinology and Laryngology* **100 (5 Pt1)**: **378-384**

Rubin BK: Surface properties of respiratory secretions: relationship to mucus transport., Cilia, Mucus, and Mucociliary Interactions. Edited by Baum GL, Marcel Dekker, New York, 1998, pp 584

Rubin BK, Finegan B, Ramirez O, King M (1990). General anesthesia does not alter the viscoelastic or transport properties of human respiratory mucus. *Chest* **98 (1)**: **101-4**

Ruiz-Santana S, Jiminez A, Esteban A, al e (1987). ICU pneumonias: A multi-institutional study. *Critical Care Medicine* **15: 930 - 932**

Rusznak C, Devalia JL, Lozewicz S, Davies RJ (1994). The assessment of nasal mucociliary clearance and the effect of drugs. *Respiratory medicine* **88: 89-101**

Rutland J, Griffin W, Cole P (1981). Nasal brushing and measurement of ciliary beat frequency. An in vitro method for evaluating pharmacologic effects on human cilia. *Chest* **80 (6 Suppl)**: **865-7**

Rutland J, Dewar A, Cox T, Cole P (1982). Nasal Brushing For the Study of Ciliary Ultrastructure. *Journal of Clinical Pathology* **35 (3)**: **357-359**

Rutland J, Cox T, Dewar A, Cole P (1983). Screening for ciliary dyskinesia - a spectrum of defects of motility and structure. *European Journal of Respiratory Diseases. Supplement* **127: 71-7**

Rutland J, de Iongh RU (1990). Random ciliary orientation. A cause of respiratory tract disease. *New England Journal Of Medicine* **323 (24): 1681-4**

Sackner MA, Landa J, Hirsch J, Zapata A (1975). Pulmonary effects of oxygen breathing. A 6-hour study in normal men. *Annals of Internal Medicine* **82 (1): 40-3**

Salathe M, Pratt MM, Wanner A (1993). Cyclic AMP dependent phosphorylation of a 26 kDA axonemal protein in ovine cilia isolated from small tissue pieces. *American Journal of Respiratory Cell and Molecular Biology* **9: 306 - 314**

Sanderson MJ, Dirksen ER (1986). Mechanosensitivity of cultured ciliated cells from the mammalian respiratory tract: implications for the regulation of mucociliary clearance. *Proc Natl Acad Sci USA* **83: 7302-7306**

Satir P (1965). Studies on cilia: II. Examination of the distal region of the ciliary shaft and the role of filaments in motility. *J. Cell Biol* **26: 805-834**

Satir P (1995). Landmarks in cilia research from Leeuwenhoek to us. *Cell Motility and the Cytoskeleton* **32: 90-94**

Selwyn DA, Gyi A, Raphael JH, Key A, Langton JA (1996). A perfusion system for in vitro measurement of human cilia beat frequency. *British Journal of Anaesthesia* **76 (1): 111-5**

Selwyn DA, Raphael JH, Lambert DG, Langton JA (1996). Effects of morphine on human nasal cilia beat frequency in vitro. *British Journal of Anaesthesia* **76 (2): 274-7**

Sharpey W: Cilia, The Cyclopedia of Anatomy and Physiology. Edited by Todd RB. London, Longman, Brown Gree, Longmans, and Roberts, 1835-36, pp 606-638 In: Satir (1995)

Shimizu T, Nishihara M, Kawaguchi S, Sakakura Y (1994). Expression of phenotypic markers during regeneration of rat tracheal epithelium following mechanical injury. *American Journal of Respiratory Cell and Molecular Biology* **11: 85 - 94**

Shirakami G, Li D, Zhan X, Johns RA (2000). Propofol stimulates ciliary motility via the nitric oxide-cyclic GMP pathway in cultured rat tracheal epithelial cells. *Anesthesiology* **93 (2)**: **482-8**

Sleigh MA (1962): The Biology of Cilia and Flagella., Pergamon Press, Oxford.

Sleigh MA, Blake JR, Liron N (1988). The propulsion of mucus by cilia. *American Review of Respiratory Disease* **137: 726-741**

Souma T (1987). The distribution and surface ultrastructure of airway epithelial cells in the rat lung: a scanning electron microscopic study. *Archivum Histologicum Japnicum* **50 (4)**: **419-436**

Spencer RC (1994). Epidemiology of infection in ICUs. *Intensive Care Medicine* **20: 52-56**

Stanek A, Brambrink AM, Latorre F, Bender B, Kleemann PP (1998). Effects of normobaric oxygen on ciliary beat frequency of human respiratory epithelium. *British Journal of Anaesthesia* **80 (5)**: **660-4**

Sturgess JM, Chao J, Turner JAP (1980). Transposition of ciliary microtubules. Another cause of impaired ciliary motility. *The New England Journal of Medicine* **303 (6)**: **319-321**

Svartengren K, Wiman LG, Thyberg P, Rigler R (1989). Laser light scattering spectroscopy: a new method to measure tracheobronchial mucociliary activity. *Thorax* **44 (7)**: **539-47**

Takasaka T, Sato M, Onodera A (1980). Atypical cilia of the human nasal mucosa. *Annals of Otology, Rhinology and Laryngology* **89 (1 Pt 1)**: **37-45**

Tamalet A, Clement A, Roudot-Thoraval F, Desmarquest P, Roger G, Boule M, Millepied MC, Baculard A, Escudier E (2001). Abnormal central complex is a marker of severity in the presence of partial cliary defect. *Pediatrics* **108 (5)**: E86

Thomson ML, Pavia D (1973). Long-term tobacco smoking and mucociliary clearance from the human lung in health and respiratory impairment. *Archives Of Environmental Health* **26 (2)**: **86-9**

Torkkeli T, Nuutinen J, Rautiainen M (1997). Clinical relevance of tubulus anomalies and compound cilia. *Acta Otolaryngol Suppl* **529: 140-3**

Toskala E, Rautiainen M, Nuutinen J (1994). Scanning and transmission electron microscopic findings in cilia from human nasal turbinate and sinus mucosa following respiratory infection. *European Archives of Oto-Rhino-Laryngology* **251 (2)**: **76-9**

Toskala E (1995). The influence of specimen preparation on artefacts in scanning electron microscopy of respiratory cilia. *Biotechnic and Histochemistry* **70 (1)**: **46-51**

Toskala E, Nuutinen J, Rautiainen M (1995). Scanning electron microscopy findings of human respiratory cilia in chronic sinusitis and in recurrent respiratory infections. *Journal of Laryngology and Otology* **109 (6)**: **509-14**

Toskala E, Nuutinen J, Rautiainen M, Pelttari A (1995). Gold coating of respiratory cilia for scanning electron microscopy. *Scanning Microscopy* **9 (1)**: **303-6**

Toskala E, Westrin KM, Stierna P, Rautiainen M (1997). Ciliary ultrastructure in experimental sinusitis. *Acta Otolaryngol Suppl* **529: 137-9**

Trevisani L, Sartori S, Bovolenta MR, Mazzoni M, Pazzi P, Putinati S, Potena A (1992). Structural characterization of the bronchial epithelium of subjects with chronic bronchitis and in asymptomatic smokers. *Respiration* **59 (3)**: **136-44**

Tsuda T, Noguchi H, Takumi Y, Aochi O (1977). Optimum humidification of air administered to a tracheostomy in dogs. Scanning electron microscopy and surfactant studies. *British Journal of Anaesthesia* **49 (10)**: **965-77**

Van der Baan S, Veerman AJP, Bezemer PD, Feenstra L (1987). Primary ciliary dyskinesia: quantitative investigation of the ciliary ultrastructure with statistical analysis. *Annals of Otology, Rhinology, and Laryngology* **96: 264-272**

Verdugo P, Golborne CE (1988). Remote detection of ciliary movement by fiber optic laser-Doppler spectroscopy. *IEEE Transactions on Biomedical Engineering* **35 (5)**: **303-7**

Verra F, Escudier E, Lebargy F, Bernaudin JF, De Crémoux H, Bignon J (1995). Ciliary abnormalities in bronchial epithelium of smokers, ex-smokers, and nonsmokers. *American Journal Of Respiratory And Critical Care Medicine* **151 (3 Pt 1)**: **630-4**

Vincent JL, Bihari DJ, Suter PM (1995). The prevalence of nosocomial infection in intensive care units in Europe. Resuts of the European Prevalence of Infection in Intensive Care (EPIC) Study. EPIC International advisory committee. *JAMA* **274: 639 - 644**

Wanner A, Hirsch JA, Greeneltch DE, Swenson EW, Fore T (1973). Tracheal mucous velocity in beagles after chronic exposure to cigarette smoke. *Archives of Environmental Health* **27 (6)**: **370-1**

Wanner A (1985). A review of the effects of cigarette smoke on airway mucosal function. *European Journal of Respiratory Diseases. Supplement* **139: 49-53**

Wanner A, Salathe M, O'Riordan TG (1996). Mucociliary clearance in the airways. *American Journal of Respiratory and Critical Care Medicine* **154 (6 Pt 1)**: **1868-902**

Weisman Z, Sadé J (1979). Effect of environmental CO2, O2 and pH on the growth of respiratory epithelium in vitro. *Annals of Otology, Rhinology and Laryngology* **88 (1 Pt 1)**: **21-30**

Westergaard O, Olsen P (1973). Smoking and ciliary movement in the upper respiratory tract. *Archiv Fur Klinische Und Experimentelle Ohren-, Nasen- Und Kehlkopfheilkunde* **203 (3)**: **179-83**

Williams R, Rankin N, Smith T, Galler D, Seakins P (1996). Relationship between the humidity and temperature of inspired gas and the function of the airway mucosa. *Critical Care Medicine* **24 (11)**: **1920-9**

Wilson DW, Plopper CG, Hyde DM (1984). The tracheobronchial epithelium of the Bonnet monkey (Macaca radiata): A quantitative ultrastructural study. *The American Journal of Anatomy* **171: 25-40**

Winn RE (1990): Respiratory infections in critical care patients., Problems in critical care. Lippincott, Philadelphia, pp 125-144

Wolfe WG, Ebert PA, Sabiston DCJ (1972). Effect of high oxygen tension on mucociliary function. *Surgery* **72 (2)**: **246-52**

Yager J, Chen TM, Dulfano MJ (1978). Measurement of frequency of ciliary beats of human respiratory epithelium. *Chest* **73 (5)**: **627-33**

Yeates D, Aspin N, levison H, Jones M, Bryan A (1975). Mucociliary tracheal transport rates in man. *Journal of Applied Physiology* **39: 487 - 495**

Yoshitsugu M, Rautiainen M, Matsune S, Nuutinen J, Ohyama M (1993). Effect of exogenous ATP on ciliary beat of human ciliated cells studied with differential interference microscope equipped with high speed video. *Acta Otolaryngologica* **113 (5)**: **655-9**

Yoshitsugu M, Hanamure Y, Furuta S, Deguchi K, Ueno K, Rautiainen M (1994). Ciliary motility and surface morphology of cultured human respiratory epithelial cells during ciliogenesis. *Biology of the Cell* **82 (2-3)**: **211-6**

Zahm JM, Lamiot E, Chevillard D, Hinnrasky J, Puchelle E (1990). Quantitation of in vitro ciliated cell growth through image analysis. *In Vitro Cellular and Developmental Biology* **26 (11)**: **1063-1067**

APPENDIX

Ethical Committee Approval

From the office of the Chairman of the
PLYMOUTH LOCAL RESEARCH ETHICS COMMITTEE
(Committee Secretary - Linda Farr -Tel: 01803 861876/Fax: 01903 [illegible]5)
(Short Code from Derriford Hospital - #6410)

Please quote Plymouth Trial No on all correspondence

South & West Devon Health AUTHORITY

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MTI/LMF

18th June 1996

Dr J Langton
Consultant Anaesthetist
Plymouth Hospitals NHS Trust
Derriford Hospital
PLYMOUTH PL6 8DH

Dear Jeremy,

Respiratory cilia in smokers - Plymouth Study No 759

Thank you for submitting your application for the above study which was discussed by the Local Research Ethics Committee on the 11th June 1996.

After some discussion, it was agreed that this was a useful way of investigating a problem which has been around for many years and the Committee was happy for you to go ahead. One Member was interested in receiving further information on this study and I hope you have been able to get in touch with her.

The Committee requests that you forward a report on progress and outcome in due course.

With all best wishes.

Yours sincerely

Michael Inman

Dr M T Inman
Chairman
Local Research Ethics Committee

From the office of the Chairman of the
PLYMOUTH LOCAL RESEARCH ETHICS COMMITTEE
(Committee Secretary - Linda Farr - Tel: 01803 861876/Fax: 01803 866425)
(Short Code from Derriford Hospital - #6410)

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MTI/LMF

17th February 1997

Dr J Langton
Consultant Anaesthetist
Plymouth Hospitals NHS Trust
Derriford Hospital
PLYMOUTH PL6 5DH

Dear Jeremy,

Respiratory Cilia in the Critically Ill - Plymouth Study No 860

Thank you for submitting your application for the above study which was considered by the Plymouth Local Research Ethics Committee on the 11th February 1997.

We were perfectly happy for the study to go ahead.

With all best wishes.

Yours sincerely

Dr M T Inman
Chairman
Local Research Ethics Committee

SOUTH AND WEST (PLYMOUTH) LOCAL RESEARCH ETHICS COMMITTEE APPLICATION FORM

For Ethics Committee use only:	*Number*	260	*Date received*	2-7-9
	Outcome		*Applicant informed*	

INSTRUCTIONS: Please complete in typescript. Please place a circle round Yes/No options as appropriate. A version of this form is also available on disc in Word for Windows from the Ethics Committee Secretary or the Regional Research and Development Directorate.

It is essential that this form is completed fully and the relevant enclosures are received if the study is to receive proper scrutiny by the Ethics Committee. Please refer to the accompanying Guidance Notes when completing the form. Please complete the checklist before sending the form.

CHECKLIST

Please indicate if the following have been enclosed by placing a circle round *Yes/No/Not applicable* options. For details of the numbers of copies of the form and relevant enclosures required, please contact the relevant LREC secretary. (See Appendix 5 in the Guidance Notes for details.)

1 copy of Application Form		(Yes)	No	Not applicable
2 copies of protocol		(Yes)	No	Not applicable
Patient consent form		(Yes)	No	Not applicable
Patient information sheet		(Yes)	No	Not applicable
GP/consultant information sheet		(Yes)	No	Not applicable
Questionnaire*	Finalised/Not yet finalised	Yes	(No)	Not applicable
Copy of manufacturers data sheet for all drugs (one copy only)		Yes	(No)	Not applicable
Copy of manufacturers indemnity (two copies)		Yes	(No)	Not applicable
Copy of CTX/CTL/DDX (one copy only)		Yes	(No)	Not applicable
Annexe A **		Yes	(No)	Not applicable
Annexe B ***		Yes	(No)	Not applicable

* *Please indicate if not yet yet finalised.*

** *If the study involves the use of a new medicinal product or medical device, or the use of an exiting product outside the terms of its produce licence, Annexe A is included in the Guidance Notes*

*** *If the study includes the use of ionising or non-ionising radiation, radioactive substances or X-Rays, Annexe B is included in the Guidance Notes.*

SECTION 1 Details of applicant(s)

1. **Short title of project** (*in not more than 6 words)*

Respiratory cilia in the critically ill

Full title : **A structural and functional investigation of respiratory ciliated epithelium in the critically ill.**

Summary of practical benefits/improvements in patient care which are envisaged:

Increasing numbers of patients are now benefiting from intensive care, however a number of common clinical problems still exist. One of these is the development of a chest infection. This has been shown to occur in up to 40% of patients admitted to the intensive care unit, this has been shown to increase mortality and length of stay. Respiratory cilia are known to be closely involved with the clearance of secretions from the lungs and impairment of cilia function predisposes to the accumulation of secretions. Retention of secretions may lead to the development of a chest infection. Despite the widespread nature of this problem there are at present no studies which have investigated respiratory cilia structure and function in the critically ill.
The aim of this study is to observe the natural history of cilia structure and function in patients on the intensive care unit. This will allow the development of a rational approach to the management of these patients and may allow earlier prediction of respiratory problems and the development of more effective treatments.

2. **Applicant** *(All correspondence will be sent to this address unless indicated otherwise)*

Surname: **LANGTON** Forename: **JEREMY** Title: **Dr**

Present appointment of applicant: Consultant Anaesthetist, Derriford Hospital, Plymouth.

Qualifications: MB,BS MD FRCA

Address: Department of Anaesthesia, Derriford Hospital, Plymouth, Devon.
Tel: 01752 792691 Ext. 53280
Fax: 01752 763287

3. **Other workers and departments/institutions involved :**

Dr R Sneyd Consultant Anaesthetist, Derriford Hospital.
Dr. P. MacNaughton Director, Intensive Care Unit, Derriford Hospital Plymouth.

There is no existing data in the literature on which to base estimates of sample size. We would initially aim to investigate samples from 30 patients.

ii) **Was there formal statistical input into the overall study design?** Yes (No)

If Yes, please give name of adviser:

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10. **Does the study fall into any of the following categories?**

Pilot	Yes	No
Multi-centre Study	Yes	No
Student project *(part of course requirement)*	Yes	No

If student project, what course is being undertaken, in which institution?

If this is a multi-centre study, please complete the details below, otherwise go to Question 11.

i) **Which centres are involved?**

ii) **Which Ethics Committees have been approached and what is the outcome to date?**

iii) **Who will have overall responsibility for the study?**

iv) **Who has control of the data generated?**